Persister formation in *Borrelia burgdorferi*, *Staphylococcus aureus*, and *Escherichia coli*

by Autumn Brown Gandt
BS in Biology, Gordon College

A dissertation submitted to

The Faculty of
the College of Science of
Northeastern University
in partial fulfillment of the requirement
for the degree of Doctor of Philosophy

April 4, 2016

Dissertation directed by
Kim Lewis
Distinguished Professor of Biology
ACNOWLEDGEMENTS

I would like to thank my advisor Kim Lewis for giving me a place in his lab and for his guidance and support throughout the duration of my PhD.

I would also like to thank my committee members Veronica Godoy, Win Chai, and Tim Van Opijnen for their thoughtful questions and helpful advice.

Thanks to all of the current and former members of the Lewis Lab. Sarah Rowe taught me everything I know about how to work in a microbiology lab. I would not have gotten to this point without her. Many thanks to Brian Conlon for his many thoughtful discussions about my projects and science in general. Austin Nuxoll has been incredibly helpful with all things related to S. aureus. Yeva Shan has been wonderful to work with. I will miss our Friday afternoon science conversations. Bijaya Sharma was my partner in crime in all things Borrelia. Thanks for being a friend inside and outside the lab. Phil Strandwitz has been an amazing resource in the lab and a great friend outside of it. Thanks to Lauren Fitch, I am glad I got to work with you! Many thanks to all of the current and former members of the Lewis Lab for making it a fun and stimulating place to work. Especially all members of the Staph and E. coli persister groups: Eliza Zalis, Austin Nuxoll, Tomoyuki Hamma, Brian Conlon, and Sarah Rowe; Sammy Nicolau, Yeva Shan, Pooja Balani, Alyssa Theodore, Sarah Rowe, Marin Viluc, and Sonja Hansen.

Thanks also to several talented undergraduates who helped me with my work and taught me how to teach: Cicely Krebill, Molly Ingemi, Martha Gowaski, Ryan Twohig, and Samantha Yao.

Thank you also to my family. My husband Jeff for his support, encouragement, and patience. Thanks for listening to me ramble about work. My mother for her support and advice over the years. My brother and sister for reminding me that there is more to life than the lab.
ABSTRACT OF DISSERTATION

Persisters are phenotypic variants within a bacterial population that can survive lethal concentrations of antibiotics. Persisters have been implicated in the recalcitrance of chronic infections. This thesis explores persister formation in three species: *Borrelia burgdorferi*, *Staphylococcus aureus*, and *Escherichia coli*. *B. burgdorferi* is the causative agent of Lyme disease. If not treated early, *B. burgdorferi* infection can result in symptoms like severe fatigue, pain, cognitive impairment, and arthritis after completion of the standard treatment regimen. *B. burgdorferi* persisters had not been characterized *in vitro*. We found that *B. burgdorferi* forms a growth-phase dependent subpopulation of persister cells tolerant to the clinically utilized antibiotics, doxycycline, amoxicillin, and ceftriaxone. These persisters can be eradicated by the DNA damaging agent mitomycin C or pulse dosing with ceftriaxone. *S. aureus* is a clinically important pathogen which forms hard to treat biofilm infections. *S. aureus* is unique because it is completely tolerant to multiple classes of antibiotics in stationary phase. Due to its high tolerance in stationary phase, we reasoned that persister formation may be triggered by environmental stimuli. Utilizing a genetic screen, we find that the two component systems and quorum sensing are not involved in persister formation in *S. aureus*. An evaluation of stresses meant to emulate stationary phase revealed that stressors which decrease cellular energy or reduce translation result in increased persister formation. We conclude that in *S. aureus* persisters are stationary-like cells with low translation and ATP levels. *E. coli* is the model organism for studying persisters. We re-examine the emerging model of persister formation and find that rather than the stringent response and toxin-antitoxin systems, persister formation is triggered by low ATP levels.
# TABLE OF CONTENTS

Acknowledgements ............................................................................................................................ ii

Abstract of Dissertation ......................................................................................................................... iii

List of Figures and Tables ......................................................................................................................... vi

Table of Abbreviations ............................................................................................................................. vii

Chapter 1: Introduction ............................................................................................................................. 1

1.1 Persisters in *Borrelia burgdorferi* ................................................................................................. 2

1.2 Persisters in *Staphylococcus aureus* ............................................................................................... 3

1.3 Persisters in *Escherichia coli* ........................................................................................................... 6

References cited: ....................................................................................................................................... 8

Chapter 2: *Borrelia burgdorferi*, the causative agent of Lyme disease, forms drug-tolerant persister cells. ............................................................................................................................... 12

ABSTRACT .............................................................................................................................................. 12

INTRODUCTION ....................................................................................................................................... 13

MATERIALS AND METHODS .................................................................................................................... 14

RESULTS .................................................................................................................................................. 17

Characterization of *B. burgdorferi* Persisters ......................................................................................... 17

Eradication of *B. burgdorferi* Persisters ................................................................................................. 21

DISCUSSION .......................................................................................................................................... 26

REFERENCES CITED ............................................................................................................................... 30

Chapter 3: Persister formation through ATP depletion and decreased translation in *Staphylococcus aureus* .................................................................................................................................... 34

ABSTRACT .............................................................................................................................................. 34
INTRODUCTION.............................................................................................................................................34

MATERIAL AND METHODS .............................................................................................................................36

RESULTS........................................................................................................................................................38

DISCUSSION..................................................................................................................................................44

FUTURE WORK...............................................................................................................................................46

REFERENCES CITED.......................................................................................................................................47

Chapter 4: Stochastic formation of drug-tolerant persisters in *Escherichia coli*...........................................50

ABSTRACT....................................................................................................................................................50

INTRODUCTION...........................................................................................................................................50

MATERIALS AND METHODS............................................................................................................................52

Results...........................................................................................................................................................56

TA interferases are upregulated by stress.......................................................................................................56

Δ10TA strain’s multi-drug low persister phenotype depends on the media used...........................................60

Δ10TA decreases persister level through a translation independent mechanism........................................62

Lon and PolyP are not involved in persister formation...............................................................................63

*rrnB P1* reports persister formation independently of TA interferases and ppGpp....................................67

A drop in intracellular ATP causes persister formation..............................................................................70

Discussion.....................................................................................................................................................71

Future Direction..........................................................................................................................................75

References cited..............................................................................................................................................76
LIST OF FIGURES AND TABLES

Chapter 1:
Figure 1: Characteristics of persister cells

Chapter 2:
Table 1: Selected antibiotics tested against B. burgdorferi.
Figure 1: Killing of B. burgdorferi by antibiotics
Figure 2: Growth dependent persister formation in B. burgdorferi
Figure 3: Persister formation is not heritable
Figure 4: Killing of B. burgdorferi with drug combinations
Figure 5: Killing of B. burgdorferi by daptomycin
Figure 6: Killing of B. burgdorferi by mitomycin C (MMC).
Figure 7: Pulse dosing results in effective killing of B. burgdorferi persisters

Chapter 3:
Figure 1: Two component systems do not contribute to persister formation in S. aureus
Figure 2: Transition to an anaerobic environment increases persister formation independently of nreC, srrB, and rex.
Figure 3: Treatment of an exponential culture with mupirocin, linezolid, or arsenate increases persister formation
Figure 4: Lowering ATP or translation to stationary levels in an exponential culture increases tolerance in an antibiotic specific manner.

Chapter 4:
Table 1: Strain list
Table 2: Primers for creation of plasmid based GFP promoter fusions
Table 3: Primers for the deletion of spoT
Figure 1: Toxin-antitoxins are induced in response to stress conditions
Figure 2: Upregulated expression of TA modules induces persister formation only under isoleucine starvation
Figure 3: Phenotype of Δ10TA is dependent on antibiotic and growth conditions
Figure 4: Induction of persisters by toxins does not depend on inhibition of translation
Figure 5: Lon and PolyP do not impact multidrug tolerance
Figure 6: Effect of relAspoT mutation on persister formation
Figure 7: Effect of SHX and isoleucine starvation on persister formation in the ΔrelAΔspoT background
Figure 8: rmB P1 promoter activity correlates with persisters and is repressed upon entrance into stationary phase independently of TA interferases and ppGpp
Figure 9: rmB P1 senses ATP level independently of ppGpp
Figure 10: Lowering intracellular ATP level leads to increased persister formation.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADEP</td>
<td>acyldepsipeptide</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>Amox</td>
<td>amoxicillin</td>
</tr>
<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>Ars</td>
<td>arsenate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSK II</td>
<td>Barbour-Stoener-Kelly II</td>
</tr>
<tr>
<td>cam</td>
<td>chloramphenicol</td>
</tr>
<tr>
<td>Cef</td>
<td>ceftriaxone</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>Cip</td>
<td>ciprofloxacin</td>
</tr>
<tr>
<td>Cipro</td>
<td>ciprofloxacin</td>
</tr>
<tr>
<td>Ctrl</td>
<td>Control</td>
</tr>
<tr>
<td>Dox</td>
<td>doxycycline</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>Gent</td>
<td>gentamicin</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>hip</td>
<td>high persister</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>Ile</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
<tr>
<td>Lin</td>
<td>linezolid</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
</tr>
<tr>
<td>MMC</td>
<td>Mitomycin C</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MSSA</td>
<td>Methicillin susceptible <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Pi</td>
<td>phosphate</td>
</tr>
<tr>
<td>RPM</td>
<td>rotation per minute</td>
</tr>
<tr>
<td>SHX</td>
<td>serine hydroxamate</td>
</tr>
<tr>
<td>Spec</td>
<td>spectinomycin</td>
</tr>
<tr>
<td>TA</td>
<td>Toxin-Antitoxin</td>
</tr>
<tr>
<td>TCS</td>
<td>Two Component System</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
</tr>
<tr>
<td>Vanc</td>
<td>vancomycin</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

Authors: Autumn Brown Gandt and Kim Lewis

Persisters are a subpopulation of bacteria that survive lethal concentrations of antibiotics. Persisters are distinct from resistant mutants; their drug-tolerance is not heritable and they cannot grow in the presence of antibiotics (Figure 1A). Persisters were first discovered by Joseph Bigger in the 1940s when he noticed that a small percentage of Staphylococcus aureus cells survived exposure to high levels of penicillin (Bigger, 1944). Since then, persisters have been identified in all species studied, including Escherichia coli, Salmonella Typhimurium, Mycobacterium tuberculosis, and Pseudomonas aeruginosa (Helaine et al., 2014; Keren et al., 2004a; Mulcahy et al., 2010; Wayne and Sohaskey, 2001).

Persisters have been implicated in the recalcitrance of chronic infections to antibiotic treatment. A study of longitudinal P. aeruginosa isolates from cystic fibrosis patients revealed that later isolates had accrued mutations which increased their persister levels. These high persister strains did not have any change in minimum inhibitory concentration (MIC) which measures resistance to an antibiotic (Mulcahy et al., 2010). In a similar study, it was found that late isolates of Candida albicans from patients with oral thrush had increased persister formation (LaFleur et al., 2010). A more recent study examined the occurrence of the high persister (hip) allele hipA7 in urinary tract infection (UTI) and commensal isolates of E. coli (Schumacher et al., 2015). This allele was found both in clinical and commensal isolates and contributed to high persister levels in these strains. These studies suggest that antibiotic treatment over time selects for strains with higher persister levels, linking persisters to clinical manifestation of disease.
Figure 1: Characteristics of persister cells. (A) The presence of persisters is indicated by biphasic killing after the addition of an antibiotic. Unlike resistant mutants, persisters do not grow in the presence of antibiotics. (B) Persister formation is growth phase dependent, with persister levels increasing throughout growth and reaching a maximum in stationary phase.

The persister subpopulation is indicated by biphasic killing pattern after a population is challenged with a bactericidal antibiotic. After the addition of an antibiotic, the bulk of the population rapidly dies, leaving a subpopulation of persisters that are killed at a slower rate (Figure 1A). Persister formation is growth phase dependent, persister levels are lowest in an actively growing culture and highest during stationary phase, reaching ~1% in *E. coli* (Figure 1B) (Keren et al., 2004a). Evidence suggests that persisters are non-growing, metabolically inactive cells (Dorr et al., 2010; Shah et al., 2006). However, some active mechanisms are needed for persister maintenance and resuscitation. The DNA damage (SOS) response, for example, is necessary for drug tolerance to the DNA damaging fluoroquinolone class of antibiotics in *E. coli* (Theodore et al., 2013).

This thesis addresses three questions about persister formation. First, the previously uninvestigated question of whether *Borrelia burgdorferi* forms canonical persister cells in vitro (Sharma et al., 2015). Second, the nature of persister formation in the Gram-positive pathogen *S. aureus*. Finally, I present a re-examination of the current widely accepted model of persister formation in *E. coli*.

### 1.1 Persisters in *Borrelia burgdorferi*

Lyme disease is the most prevalent vector borne disease in the United States. In North America, it is caused by the spirochete *B. burgdorferi* and is transmitted by ticks of the *Ixodes* genus. If treated
early, most cases of Lyme disease resolve. However, 10-30% of patients report long-term symptoms after antibiotic treatment (Dersch et al., 2016; Weitzner et al., 2015). This is called Post Treatment Lyme Disease Syndrome (PTLDS). The cause of these unresolved symptoms is a matter of contentious debate in the field (Halperin et al., 2013). Some posit that PTLDS is the result of an autoimmune reaction or an immune response to latent *Borrelia* antigens (Bolz and Weis, 2004). Others believe that there is still active *Borrelia* infection after antibiotic treatment (Embers et al., 2012). If Lyme disease is not treated early, severe symptoms such as arthritis, neurological disorders, and intense fatigue can develop.

Several studies have investigated whether *B. burgdorferi* is cleared after antibiotic treatment in mouse and monkey models of Lyme disease. In a mouse model of Lyme disease it was found that after antibiotic treatment, tissues were PCR negative for *B. burgdorferi* for several months but then positive at one year post treatment (Hodzic et al., 2014). This suggests a resurgence of bacteria after treatment has ceased. A similar study in rhesus macaques showed that an infection with *B. burgdorferi* is not always cleared by treatment with either ceftriaxone or doxycycline, two antibiotics used clinically to treat Lyme disease (Embers et al., 2012). The presence of *B. burgdorferi* in this study was indicated by a positive result in a PCR, RT-PCR, xenodiagnosis, or immunofluorescence assay.

These results, as well as the possible role of antibiotic failure in PTLDS, prompted an interest in persister cells in *B. burgdorferi* (Caskey and Embers, 2015; Feng et al., 2014). However, it had not been established whether *B. burgdorferi* forms traditional persister cells in vitro. In our study (Sharma et al., 2015), we find that *B. burgdorferi* shows biphasic killing after the addition of antibiotics, an indication of the presence of persister cells. These persisters can survive very high doses of antibiotics, far beyond what is clinically achievable. As in other species, persister formation in *B. burgdorferi* is growth phase dependent. After establishing that *B. burgdorferi* forms persisters in vitro, we sought a way to eradicate them. We found that either treatment with the DNA damaging agent mitomycin C or pulse dosing with ceftriaxone can sterilize a *B. burgdorferi* population in vitro.

### 1.2 Persisters in *Staphylococcus aureus* 

*S. aureus* is an important human pathogen. It is associated with hospital acquired infections in immune compromised patients and, increasingly, highly virulent community acquired infections in healthy
individuals (Otto, 2007). S. aureus forms biofilm-based infections including osteomyelitis, endocarditis, and biofilms on indwelling devices such as catheters and prostheses (Conlon, 2014). Biofilm infections are notoriously hard to treat successfully (Archer et al., 2011). The exopolymer matrix of the biofilm protects pathogens from immune cells (Jesaitis et al., 2003; Leid et al., 2002; Vuong et al., 2004). While antibiotics can penetrate biofilm to kill active cells, persisters survive (Spoering and Lewis, 2001). Biofilms act as a reservoir for antibiotic tolerant persister cells which can restart the infection after completion of antibiotic treatment (Lewis, 2010).

S. aureus is highly tolerant to multiple classes of antibiotics in stationary phase, reaching 100% persisters tolerant to beta lactams, fluoroquinolones, the glycopeptide vancomycin, and the RNA polymerase targeting antibiotic rifampicin (Conlon et al., 2013; Keren et al., 2004a). Beta-lactams and vancomycin kill by corrupting cell wall synthesis, and therefore only kill when cells are actively making peptidoglycan during growth and division (Kohanski et al., 2010). Tolerance of non-growing cells is therefore not surprising and complete tolerance to these growth dependent antibiotics in stationary phase is present in multiple species including S. aureus.

In Gram-positive bacteria, fluoroquinolones target topoisomerase IV, which cuts and rejoins DNA to relax supercoiling during transcription and replication. Fluoroquinolones bind to topoisomerase IV and prevent strand rejoining, causing double strand breaks which accumulate and kill the cell (Kohanski et al., 2010). This mechanism of action does not require active growth and fluoroquinolones can readily kill stationary phase cells of multiple species (Keren et al., 2004a). However, fluoroquinolones do not kill stationary phase S. aureus (Conlon et al., 2013).

Some classes of antibiotics used at high levels are able to kill stationary phase S. aureus. These antibiotics have mechanisms of action that allow them to kill even inactive persister cells. Daptomycin, for example, effectively kills stationary phase S. aureus, leaving a small subpopulation of persisters (Lechner et al., 2012). Daptomycin is a lipopeptide antibiotic that targets the cell membrane, creating membrane pores. This mechanism of action is independent of active cellular processes allowing daptomycin to kill otherwise drug-tolerant stationary cells.

Understanding the dormant nature of persister cells has allowed for the design of more effective therapies. For example, potentiating aminoglycosides with certain sugars increased the energy-
dependent uptake of aminoglycosides and resulted in greater killing of *S. aureus* (Allison et al., 2011). Acyldepsipeptides (ADEPs) are antibiotics which activate the protease ClpP, allowing it to degrade proteins in an ATP-independent manner (Kirstein et al., 2009). This leads the cells to self-digest and die. ADEP4, in combination with rifampicin (to prevent the growth of ADEP resistant mutants which occur at a frequency of $10^{-6}$), is able to sterilize *S. aureus* both *in vitro* and in an *in vivo* biofilm model of infection (Conlon et al., 2013).

The molecular mechanism of persister formation in *S. aureus* is poorly understood. The toxin-antitoxin systems and the stringent response, which have been linked to persister formation in *E. coli*, are not involved in persister formation in *S. aureus* (Conlon et al., 2016). The fact that stationary cells are completely tolerant to many antibiotics may give a clue to the mechanism of persister formation in *S. aureus*. Based on this knowledge, I used a two pronged approach to study the mechanism of persister formation in *S. aureus*. First, I performed a targeted genetic screen of the two component systems (TCSs) because of their involvement in responding to stress stimuli. TCSs consist of a membrane bound histidine kinase and a cytoplasmic response regulator. The histidine kinase senses an external or internal stimuli such as oxygen concentration, cell wall stress, phosphate availability, or cell membrane electrical potential (Freeman et al., 2013; Hall and Ji, 2013; Kawada-Matsuo et al., 2013; Yang et al., 2013). When activated, the histidine kinase phosphorylates its corresponding response regulator. The response regulator is a transcriptional regulator that activates or represses genes in order to respond to the stimuli. Included in the TCSs of *S. aureus* is the major quorum sensing and virulence regulation system *agr*. The *agr* systems was a particularly interesting persister gene candidate because both quorum sensing and persister formation are turned on at high cell density. In addition, quorum sensing has been linked to persister formation in other species (Leung and Levesque, 2012; Que et al., 2013). I found that the two component systems and quorum sensing are not involved in persister formation in *S. aureus* under regular growth conditions.

Second, I considered the conditions that are different in stationary phase and exponentially growing cultures and tested the effect of these stresses on persister formation. Lowered oxygen and induced amino acid starvation both increased persister formation. Both translation and ATP levels are lowered in stationary phase. Lowering ATP level or translation rate in exponential cells increased drug
tolerance. Coupled with concurrent work being performed in the lab (Conlon et al., 2016), we conclude that *S. aureus* persisters present in exponential phase are stationary-like cells that have low ATP levels. Low ATP is a satisfactory explanation for persister formation. As discussed above, killing by many antibiotic classes require active cellular targets. In a low ATP cell, these targets are presumably inactive, resulting in tolerance.

### 1.3 Persisters in *Escherichia coli*

Persisters formation is most well studied in the model species *E. coli*. Several genetic screens have revealed that there is no one genetic mechanism for persister formation, instead there are many redundant ways a cell can become a persister. A screen of the ordered deletion Keio collection found that deletion of chaperones (*dnaJ* and *dnaK*) and global regulators (*hns*, *hnr*, and *fis*) reduced persister formation (Hansen et al., 2008). These genes, when deleted, have pleiotropic impact on the strain, emphasizing that there is no single gene controlling persister formation. A screen of an overexpression library showed that genes involved in central metabolism (*glpD* and *plsB*) affect tolerance to beta lactams and fluoroquinolones (Spoering et al., 2006). A Tn-seq study revealed that central metabolism genes are also important for tolerance to aminoglycosides (Shan et al., 2015). These results point to the importance of central metabolism and energy generation in multidrug tolerant persister formation.

Toxin-antitoxin (TA) systems have also been linked to persister formation in *E. coli* in several studies (Dorr et al., 2010; Keren et al., 2004b; Maisonneuve et al., 2011; Moyed and Bertrand, 1983). TA systems are ubiquitous in prokaryotes. They were initially discovered as addictive genetic elements on plasmids, where they ensured plasmid maintenance by post segregational killing (Yamaguchi and Inouye, 2011). Subsequently TA systems have been found to also reside on the chromosome where their role in cell physiology is less clear. Chromosomally encoded TA systems been found in nearly every species studied to date, though, notably, not in *B. burgdorferi* (Pandey and Gerdes, 2005). Some species, such as *Mycobacterium tuberculosis*, have more than 80 putative chromosomally encoded TA loci while others have fewer than three (Leplae et al., 2011; Ramage et al., 2009).

TA systems are classified as type I, II, or III, depending on the composition of the antitoxin. In all cases, the toxin is a protein, in type I and III, the antitoxin is an RNA which is an antisense regulator of the
toxin’s expression or binds directly to the toxin to interrupt its function, respectively (Yamaguchi and Inouye, 2011). In type II TA systems, both the toxin and antitoxin are proteins. During normal growth the stable toxin is bound to its cognate labile antitoxin and inactive. During cellular stress, the antitoxin is degraded by a protease – Lon in *E. coli* – and the toxin is free to act in the cell, causing growth arrest or cell death. Toxins generally target essential cellular processes, such as DNA replication, translation, cell division, or ATP synthesis (Yamaguchi et al., 2011).

Moyed and colleagues (Moyed and Bertrand, 1983) performed the first genetic studies of persister formation. Through a screen of mutagenized *E. coli* strains they discovered that the *hipA* allele increases persister formation 100-1000 fold. HipA is a toxin of the type II TA pair *hipAB*. When released from its cognate antitoxin, HipA phosphorylates GltX, the glutamyl tRNA synthetase. Phosphorylated GltX no longer functions, resulting in uncharged tRNA\textsuperscript{eu} and a halt in protein synthesis and persister formation (Germain et al., 2013). It was recently found that the *hipA* allele increases persister formation by interrupting binding between HipA and HipB, resulting in more free toxin in the cell (Schumacher et al., 2015).

In addition to *hipAB*, several other TA systems have been implicated in persister formation. The type I TA system TisB/istR is activated by the SOS response. TisB forms a membrane pore, dissipating proton motive force and creating low energy persister cells (Dorr et al., 2010). In a transcriptomic analysis of persister cells, persisters showed increased expression of TA interferases (Keren et al., 2004b). The interferases are a group of type II TAs where the toxin is an mRNA endonuclease. There are 10 of these systems in *E. coli*. Ectopic overexpression of any interferase leads to increased persister formation (Keren et al., 2004b; Maisonneuve et al., 2011; Shah et al., 2006). It was recently shown that this is through lowered energy due to futile RNA cycling (Mok et al., 2015). Deletion of any one interferase has no effect on persister formation. However, deletion of six or more TA interferases decreases persister formation up to 200 fold (Maisonneuve et al., 2011).

Recently, a model of persister formation in *E. coli* has emerged (Germain et al., 2015; Maisonneuve et al., 2013). It has been widely accepted as the primary method of stochastic persister formation in *E. coli* (Gaca et al., 2015; Helaine and Kugelberg, 2014). This model proposes that production of the stringent response alarmone ppGpp activates the protease Lon through polyphosphate
production. Activated Lon degrades the antitoxins of TA systems, resulting in free interferase toxins. The toxins degrade mRNA, leading to slowed translation, cell dormancy, and persister formation.

Some recent reports have called into question parts of this model (Chowdhury et al., 2016). Chowdhury and colleagues show that persister formation can be induced in the absence of ppGpp by overexpression of several genes that slow growth. They argue that there are many ppGpp independent mechanisms of persister formation in \textit{E. coli}. Varik and colleagues (Varik et al., 2016) studied \textit{relA} mutant strains which are unable to produce ppGpp in response to amino acid starvation. They found that persister formation in these strains varied based on the media, and a \textit{relA} mutant actually had morePersisters under certain conditions. A study in our lab showed that the low persister phenotype of a \textit{lon} mutant was due to the action of the cell division inhibitor SulA (Theodore et al., 2013).

Our study aimed to systematically re-evaluate the stringent response-toxin-antitoxin interferase model of persister formation. We find that interferases are upregulated by several stresses. Upregulation of up to eight toxins under stress does not result in increased persister formation. Upregulation of all ten interferases by isoleucine starvation increases persister formation in an antibiotic specific manner. Further, we confirm that the phenotype of the \textit{lon} mutant is dependent on the SOS induced cell division inhibitor SulA, not the TA modules. A ppGpp$^0$ strain has a persister phenotype which is cell density dependent. Amino acid starvation increases persister formation independent of ppGpp. Using the growth phase and ATP reporter \textit{rrnB} P1, we sort stationary-like cells from an exponential population and find they are enriched for persisters. We conclude that, like in \textit{S. aureus}, lowered ATP is a cause for persister formation in \textit{E. coli}.

\textbf{Contributions:} ABG wrote the chapter. KL provided a critical reading of the chapter.

\textbf{REFERENCES CITED:}


CHAPTER 2: *Borrelia burgdorferi*, the causative agent of Lyme disease, forms drug-tolerant persister cells.

Authors: Bijaya Sharma*¹, Autumn V. Brown*¹, Nicole E. Matluck¹, Linden T. Hu² and Kim Lewis¹

*BS and AVB contributed equally to this work.

¹ Antimicrobial Discovery Center and Department of Biology, Northeastern University, Boston, Massachusetts

² Department of Medicine, Division of Geographic Medicine and Infectious Diseases, Tufts Medical Center, Boston, Massachusetts, USA

This work was published in August 2015 in Antimicrobial Agents and Chemotherapy, Volume 59, Issue 8, pages 4616–4624.

ABSTRACT

*Borrelia burgdorferi* is the causative agent of Lyme disease, which affects an estimated 300,000 people annually in the US. When treated early, the disease usually resolves, but left untreated, can result in symptoms such as arthritis and encephalopathy. Treatment of the late stage disease may require multiple courses of antibiotic therapy. Given that antibiotic resistance has not been observed for *B. burgdorferi*, the reason for the recalcitrance of late stage disease to antibiotics is unclear. In other chronic infections, the presence of drug-tolerant persisters has been linked to recalcitrance of the disease. In this study, we examined the ability of *B. burgdorferi* to form persisters. Killing of growing cultures of *B. burgdorferi* with antibiotics used to treat the disease was distinctly biphasic, with a small subpopulation of surviving cells. Upon regrowth, these cells formed a new subpopulation of antibiotic-tolerant cells, indicating that these are persisters rather than resistant mutants. The level of persisters increased sharply as the culture transitioned from exponential to stationary phase. Combinations of antibiotics did not improve killing. Daptomycin, a membrane-active bactericidal antibiotic, killed stationary phase cells, but not persisters. Mitomycin C, an anti-cancer agent that forms adducts with DNA, killed persisters and
eradicated both growing and stationary cultures of *B. burgdorferi*. Finally, we examined the ability of pulse-dosing an antibiotic to eliminate persisters. After addition of ceftriaxone, the antibiotic was washed away, surviving persisters were allowed to resuscitate, and antibiotic was added again. Four pulse-doses of ceftriaxone killed persisters, eradicating all live bacteria in the culture.

**INTRODUCTION**

All pathogens studied to date form persisters, dormant variants of regular cells which are tolerant to killing by antibiotics. The ability to produce persisters explains the puzzling recalcitrance of chronic infections to antibiotics that are effective against the same pathogen *in vitro*. Indeed, many chronic infections are caused by drug-susceptible pathogens (Burns et al., 1999; Lewis, 2010). The immune system can effectively remove sessile cells from the blood and many of the tissues, and this accounts for the efficacy of antibiotics, including bacteriostatic compounds, in treating uncomplicated infections. When the immune response is limited, the result is often a chronic infection (Lewis, 2010). Biofilms are a well-studied case of immune evasion and serve as a paradigm for understanding chronic infections. In biofilms, cells are protected from the large components of the immune system by a surface exopolymer (Jesaitis et al., 2003; Leid et al., 2002; Vuong et al., 2004). Antibiotics kill the regular cells, but dormant persisters survive, and when the concentration of antibiotic drops, they resuscitate and repopulate the biofilm (Lewis, 2010). This scenario is supported by our finding of high-persister (hip) *Pseudomonas aeruginosa* selected in the course of prolonged antibiotic treatment (Mulcahy et al., 2010). Isolated from patients with late-stage cystic fibrosis, hip mutants can produce 1000 times more persisters than the parent strain; this indicates that selection for increased tolerance (rather than resistance) provided the pathogen with a survival advantage. Similarly, hip mutants are selected during treatment of oral thrush caused by *Candida albicans* (LaFleur et al., 2010). In *Salmonella typhimurium*, entrance of pathogens into human cells where they are protected from the immune system is accompanied by a sharp increase in persister formation and tolerance to killing by antibiotics (Helaine et al., 2014). In tuberculosis, dormant cells are likely responsible for the need of a lengthy treatment of the acute stage and for the latent form of the disease. *Mycobacterium tuberculosis* hides from the immune system in macrophages or in granulomas (Barry et al., 2009).
B. burgdorferi causes Lyme disease with 300,000 estimated cases annually in the United States alone (Hinckley et al., 2014). When treated early with antibiotics, the disease usually resolves (Hu, 2012; Steere, 2001). If treatment is delayed, the pathogen spreads throughout the body and can cause meningitis, arthritis, and carditis. Meningitis and carditis are mostly self-limited, but Lyme arthritis can persist for years (Puius and Kalish, 2008; Steere et al., 1987). A substantial proportion of patients receiving their first course of antibiotics for Lyme arthritis do not respond fully to a 28 day course of treatment. In such cases, retreatment with additional courses of antibiotics is recommended (Puius and Kalish, 2008; Steere and Angelis, 2006; Wormser et al., 2006). B. burgdorferi avoids immune attack by antigenic variation of surface components and by decreasing exposure of antigens (Coutte et al., 2009; Liang et al., 2004; Radolf et al., 2012). In this regard, Lyme disease resembles other chronic infections where the pathogen is protected from the immune system, and persister cells may enable it to survive treatment with antibiotics. In Escherichia coli, the model organism for the study of persisters, dormant cells are formed primarily through expression of toxin/antitoxin (TA) modules. Toxins confer dormancy by either inhibiting protein synthesis or by decreasing the energy level of cells (Dorr et al., 2010; Germain et al., 2013; Maisonneuve et al., 2011). TA modules are widely spread among bacteria, and are copiously present in some pathogens. E. coli has more than 30 TA modules and M. tuberculosis over 75 (Sala et al., 2014; Yamaguchi and Inouye, 2011). Interestingly, there are apparently no TA modules in the genome of B. burgdorferi (Pandey and Gerdes, 2005). Virtually nothing is known about persisters in this species. In this study, we report formation of drug-tolerant persisters in B. burgdorferi and describe possible approaches to their elimination.

MATERIALS AND METHODS

Bacterial strains and growth conditions: Borrelia burgdorferi B31 5A19 that had been passaged five times in vitro was kindly provided by Dr. Monica Embers (Purser and Norris, 2000). B. burgdorferi was grown in BSK-II liquid media in a microaerophilic chamber (34°C, 3% O₂, 5% CO₂). Cultures were started by thawing -80°C glycerol stocks of B. burgdorferi (titer approximately 10⁷ cfu/mL) and diluting 1:20 into fresh BSK-II media.
BSK-II liquid medium was prepared according to protocol received from Monica Embers’ lab by adding the following ingredients to 400 ml of deionized water and mixing thoroughly: 20 g bovine serum albumin (Sigma), 2 g neopeptone (Fluka), 0.8 g yeastolate (BD), 4 g HEPES sodium salt (Sigma), 2.4 g 10X CMRL (US Biologicals), 0.28 g sodium citrate (Fisher), 0.32 g sodium pyruvate (Sigma), 2 g glucose (Fisher), 0.16 g N-acetyl-glucosamine (Sigma), 0.88 g sodium pyruvate (Sigma). The pH of the medium was adjusted to 7.6 and 24 ml of rabbit serum (Sigma) was added to the media. The medium was then filtered through a 0.22 µm filter.

Semi-solid plating was used to obtain cfu counts (Samuels, 1995). First, BSK 1.5X medium for semi-solid plating was prepared as in Samuels 1995 (Samuels, 1995). The following ingredients were added to 1 L of deionized water (LabChem, Inc) and mixed thoroughly: 8.33 g neopeptone (Fluka), 4.22 g yeastolate (BD), 9.99 g HEPES acid (Fisher), 8.33 g glucose (Fisher), 1.22 g sodium citrate (Fisher), 1.33 g sodium pyruvate (Sigma), 0.670 g N-acetyl-glucosamine (Sigma), 7.66 g sodium bicarbonate (Sigma). The pH of the media was adjusted to 7.5 and then 83.25 g of bovine serum albumin (Sigma) was added. The medium was stirred for one hour then filtered using a 0.22 µm filter. 1.5X BSK-II was stored at 4°C and used within 7 days of preparation. On the day of plating, 125 ml of 1.5X BSK was mixed with 6 ml rabbit serum and 19 ml 1X CMRL (97.89 mg/mL) and equilibrated to 55°C. 1.7% agarose (Lonza) was melted and equilibrated to 55°C. When all ingredients had equilibrated to 55°C, 1.7% agarose was added to 1.5X BSK at a ratio of 2:1 (BSK:agarose) to create BSK agarose. 8 ml of BSK agarose was dispensed into 60mm Petri dishes as bottom agar and allowed to solidify. For top agar, 100 µl of the given dilution of *B. burgdorferi* was mixed with 5 ml of 55°C BSK agarose and poured onto the bottom agar plates and allowed to solidify. The plates were incubated in zip lock bags in microaerophilic chamber (34°C, 3% O₂, 5% CO₂) for at least 21 days to obtain visible colonies.

**Antimicrobial agents:** Amoxicillin (Sigma), doxycycline hydrochloride (MP Biomedicals), ceftriaxone disodium salt hemi (heptahydrate) (Sigma), and vancomycin hydrochloride (Sigma) were dissolved in water. Mitomycin C (Sigma), gemifloxacin mesylate (Tecoland Corporation), and spectinomycin dihydrochloride pentahydrate (RPI) were dissolved in DMSO. Daptomycin cyclic lipopeptide (Sigma) was
dissolved in a 5 µg/ml solution of calcium chloride. Stock solutions of antibiotics were aliquotted and stored at -20°C until use. Antibiotics did not undergo freeze-thaw cycles.

**Killing experiments:** *B. burgdorferi* was cultured in liquid BSK-II media for 3 days to late-exponential growth phase or for 5 days to stationary phase. Antibiotics were then added to the culture. The cultures were incubated in the microaerophilic chamber (34°C, 3% O₂, 5% CO₂). At a given time point, an aliquot of the culture was washed twice by centrifuging the culture at 13.2k rpm for 5 minutes and resuspending the pellet in an equal volume of fresh BSK-II medium. The cultures were then serially diluted in fresh BSK-II media. 100 µl of the appropriate dilution was mixed with 5 ml of BSK agarose and poured as top agar. Plates were incubated in the microaerophilic chamber until visible colonies appeared (at least 21 days).

**Growth-persister experiments:** Cultures of *B. burgdorferi* were started as described above. At each time point, an aliquot of a growing culture was removed, diluted, and plated for cfu counts to generate the growth curve. A second aliquot (1 mL or 3 mL) was removed at the same time and challenged for five days with the indicated antibiotic. After five days, an aliquot of challenged culture was removed, washed twice, diluted, and plated for cfu counts to generate the persister curve.

**Minimum Inhibitory Concentration (MIC) testing:** A slightly modified version of the broth microdilution (Dever et al., 1992) was used. *B. burgdorferi* was grown in liquid culture for three days to reach exponential phase and then back diluted 1:10 into fresh BSK-II media to make the inoculum solution. All antibiotics were prepared as stock solutions in solvent (water or DMSO) based on the concentration to be tested and diluted in two fold increments in a 96 well stock plate. 2 µl per well of the antibiotic stock solution was transferred to the 96 well MIC plate to which 198 µl of the *B. burgdorferi* inoculum solution was added (final inoculum of approximately 10⁶ cells/well). Media, growth, and vehicle controls were included on each plate. The MIC plate was covered with Breatheasy Film (Diversified Biotech) and incubated in the microaerophilic chamber (34°C, 3% O₂, 5% CO₂) for 72 hours. The lowest concentration
of antibiotics that showed inhibition of growth was interpreted as the MIC. All MIC assays were repeated at least twice in triplicate.

RESULTS

Characterization of *B. burgdorferi* Persisters

The presence of persisters is indicated by a biphasic killing pattern in a time-dependent killing experiment. The bulk of the population is rapidly killed, followed by a slower rate of death in a subpopulation of tolerant cells (Lewis, 2007; Maisonneuve and Gerdes, 2014). In order to determine whether *B. burgdorferi* forms persisters, time-dependent killing experiments were performed with antibiotics commonly prescribed to patients with Lyme disease. Doxycycline is a bacteriostatic protein synthesis inhibitor; amoxicillin and ceftriaxone inhibit bacterial cell wall synthesis and are bactericidal for many bacteria. Minimum inhibitory concentrations (MICs) of doxycycline, amoxicillin, and ceftriaxone were determined (Table 1). Levels of antibiotics close to what is achievable with standard clinically prescribed treatment dosing were chosen to evaluate persister formation in *B. burgdorferi*, and we used colony forming unit (cfu) count to determine viability.
Table 1: Selected antibiotics tested against *B. burgdorferi*.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Class</th>
<th>MIC (µg/ml)*</th>
<th>Max. serum concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>β-lactam</td>
<td>0.06</td>
<td>7.6 (Gordon et al., 1972)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>Cephalosporin</td>
<td>0.01</td>
<td>256.9 (Patel et al., 1981)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>Tetracycline</td>
<td>0.25</td>
<td>2.6-5.9 (Agwuh and MacGowan, 2006)</td>
</tr>
<tr>
<td>Gemifloxacin</td>
<td>Fluoroquinolone</td>
<td>0.125</td>
<td>2.33 (Gee et al., 2001)</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>Aminoglycoside</td>
<td>2</td>
<td>140-160 (Wagner et al., 1968)</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>Lipopeptide</td>
<td>12.5-25</td>
<td>55-133 (Dvorchik et al., 2003)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>Glycopeptide</td>
<td>0.25</td>
<td>40 (Rybak, 2006)</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>Antitumor antibiotic</td>
<td>0.2</td>
<td>3.2 (den Hartigh et al., 1983)</td>
</tr>
</tbody>
</table>

*The MIC was determined by broth microdilution method.

Amoxicillin (6 µg/ml, 100X MIC) and ceftriaxone (3 µg/ml, 300X MIC) at clinically achievable levels killed the majority of cells in the first day, after which a slow phase of death followed for the next 6 days (Figure 1a). This characteristic biphasic pattern of killing is consistent with the presence of drug-tolerant persister cells.
Previous studies have shown that the persister fraction in other bacteria remains relatively unchanged even as the antibiotic level increases. We sought to determine if *B. burgdorferi* persisters behaved similarly in a dose-dependent killing experiment. As the concentration of amoxicillin and ceftriaxone increased, the fraction of surviving cells remained largely unchanged (Figures 1b-c). Doxycycline is a bacteriostatic antibiotic, but at higher concentrations appeared to effectively kill *B. burgdorferi* (Figure 1d). Again, the fraction of surviving cells did not change significantly with increasing levels of the compound. Thus, *B. burgdorferi* forms persisters capable of surviving very high concentrations of antibiotics, which exceed what is clinically achievable.
Density-dependent formation is a common feature of persisters reported for all pathogens examined so far, including *E. coli*, *Staphylococcus aureus*, *P. aeruginosa*, and *M. tuberculosis* (Conlon, 2014; Keren et al., 2011; Mulcahy et al., 2010; Norton and Mulvey, 2012). In order to test this property in *B. burgdorferi*, samples from a growing culture were removed over time, exposed to a lethal dose of antibiotic for 5 days, and then plated for cfu. There was a characteristic dip in persister levels in the early log phase, which is probably due to the resuscitation of dormant cells carried over from the inoculum (Figure 2). At mid-log phase, there is a sharp increase in persister levels, which continues as the density of the culture rises. In *E. coli*, once the culture reaches stationary state, complete tolerance is achieved for β-lactams that only kill growing cells (Tuomanen et al., 1986). In *B. burgdorferi*, we observe a very different picture – both amoxicillin and ceftriaxone kill stationary cells fairly well, yet the fraction of persisters continues to increase. One possibility is that this “stationary” culture actually represents a steady state where some cells die and others grow.

**Figure 2:** Growth dependent persister formation in *B. burgdorferi*. Growth in BSK-II medium was determined by cfu count. Persister levels were determined by taking samples from the growing culture, exposing to antibiotic for 5 days, and counting cfu. (a), amoxicillin (Amox) (6 µg/ml); (b), ceftriaxone (Cef) (3 µg/ml). N=6. Error bars represent standard error.

Next, we tested whether the *B. burgdorferi* cells surviving antibiotic treatment are drug-tolerant persisters or resistant mutants. For this, colonies produced by the surviving cells were regrown and tested for MIC. The amoxicillin and ceftriaxone MIC remained unchanged, showing that surviving cells had not acquired or developed a genetic mechanism for antibiotic resistance. The population grown from the
surviving cells produced the same level of persisters as the original population (Figure 3). These experiments show that *B. burgdorferi* forms typical persister cells.

**Figure 3: Persister formation is not heritable.** Colonies recovered from a persister experiment before and after antibiotic treatment were used to inoculate fresh BSK-II media. The colonies were allowed to grow for 3 days and treated with the same antibiotic used in the original persister experiment for 5 days. Persister levels of the colonies recovered after antibiotic treatment (Persisters) were not significantly different than the colonies recovered before antibiotic treatment (Control). N=5. Error bars represent standard error. Amox = amoxicillin, Cef = ceftriaxone.

**Eradication of *B. burgdorferi* Persisters**

**Drug combinations.** Some antibiotics act synergistically, such as sulfonamide and trimethoprim, polymixin and gentamicin, aminoglycosides and β-lactams (Levin and Harris, 1975), and we wanted to see if a combination of compounds known to be active against *B. burgdorferi* will increase efficiency of killing both regular and persister cells.

All possible two-drug combinations of amoxicillin, ceftriaxone, and doxycycline were tested with a late-exponential phase culture in a time-dependent killing experiment and found to be no more effective than the drugs used individually in killing of *B. burgdorferi* (Figure 4a). Doxycycline actually inhibited the
action of amoxicillin. We have shown previously that fluoroquinolones and aminoglycosides can kill non-growing cells (Keren et al., 2004; Spoering and Lewis, 2001), and we next tested these compounds against *B. burgdorferi*. The pathogen is generally poorly susceptible to compounds from these classes. However, the *B. burgdorferi* MICs for gemifloxacin (fluoroquinolone) and spectinomycin (aminoglycoside) are within achievable human dosing levels so we chose to test them (Gee et al., 2001; Hunfeld et al., 2000; Kraiczy et al., 2001; Wagner et al., 1968) (Table 1). Both gemifloxacin and spectinomycin were ineffective in killing *B. burgdorferi* at tested concentrations (Figure 4b). Combining these compounds also did not improve killing (Figure 4b).

**Figure 4:** Killing of *B. burgdorferi* with drug combinations. (a) Time-dependent killing of late exponential *B. burgdorferi* cultures exposed to the indicated antibiotics in combination. Amoxicillin (Amox) (6 µg/ml), ceftriaxone (Cef) (3 µg/ml), and doxycycline (Dox) (2.5 µg/ml) (n=6). (b) Killing of late exponential *B. burgdorferi* exposed to gemifloxacin (Gemi) (1.5 µg/ml) and/or spectinomycin (Spec) (160 µg/ml) singly or in combination (n=6). An aliquot was taken at indicated time points, washed, diluted, and plated on semi-solid BSK-II media for cfu counts. Error bars represent standard error.

**Experimental compounds.** Having shown that combinations of clinically prescribed antibiotics for Lyme disease are unable to effectively kill persister bacteria, we sought to examine some novel potential antimicrobial agents. We recently showed that acyldepsipeptide (ADEP4), an activator of the ClpP protease, effectively kills persisters in *S. aureus* (Conlon et al., 2013). In the presence of ADEP4, the protease cleaves mature proteins, forcing the cell to self-digest. However, ADEP4 did not have significant activity against *B. burgdorferi* (not shown), which may be due to poor penetration.
We then considered whether knowledge of *B. burgdorferi* biology might be exploited to predict vulnerability to existing approved compounds. *B. burgdorferi* lives under microaerophilic conditions, where the capacity for energy generation is limited by comparison to aerobic organisms. Daptomycin is the only approved membrane-acting antibiotic that disrupts the proton motive force. The *B. burgdorferi* MIC to daptomycin was fairly high, 12-25 µg/ml (Table 1), in accordance with published data (Feng et al., 2014). Daptomycin was highly bactericidal against *B. burgdorferi*, but a remaining subpopulation of persisters survived (Figure 5), suggesting that *B. burgdorferi* persisters can tolerate a drop in the energy level. Next, we tested vancomycin. This large glycopeptide antibiotic binds to lipid II, precursor of peptidoglycan, on the outside of the cytoplasmic membrane. Vancomycin is highly effective against Gram-positive bacteria, but does not penetrate across the outer membrane of Gram-negative species. Surprisingly, the vancomycin MIC with *B. burgdorferi* is low, 0.25 µg/ml, similarly to Gram-positive species. *B. burgdorferi* has an outer membrane; the basis for this anomaly is unclear. Vancomycin effectively killed growing cells of *B. burgdorferi*, but not persisters, and was comparable to ceftriaxone (not shown). We also tested teixobactin, a compound we recently discovered, which also binds lipid II (Ling et al., 2015). At 1.2 kDa, teixobactin is considerably smaller than vancomycin (1.8 kDa), but it did not exhibit good activity in killing *B. burgdorferi* (not shown).

**Prodrugs.** Growth under microaerophilic conditions suggests vulnerability to compounds whose action depends specifically on a low oxygen environment. Nitroaromatic compounds such as metronidazole are prodrugs that are converted into reactive drugs by bacterial nitroreductases. These enzymes are
expressed under anaerobic or microaerophilic conditions, and target pathogens living in these environments (i.e. *Helicobacter pylori*, *Clostridium difficile*, *E. coli*). We found that some nitroaromatic compounds like nitrofurantoin are effective in killing *E. coli* persisters (Fleck et al., 2014). However, we did not detect homologs of nitroreductases in the genome of *B. burgdorferi*. The MIC for nitroaromatic compounds (nitrofurantoin, nitrofurazone, and metronidazole) was too high to make them useful agents for killing *B. burgdorferi* persisters (data not shown).

Another compound that depends on a reductive environment for action is the prodrug mitomycin C. Upon entering the cell, mitomycin C is reduced into an active drug which then forms covalent adducts with DNA (Keller et al., 2001). Originally discovered in a screen for antibiotics, mitomycin C is now used as an anticancer agent. Cancers often create a microaerophilic environment, which, together with rapid cell division, accounts for the relatively selective action of mitomycin C against them. Functional RecBC and RecFOR pathways are required to repair DNA damaged by mitomycin C in *E. coli* (Keller et al., 2001). Interestingly, according to genomic data, *B. burgdorferi* lacks the genes of the RecFOR pathway (Fraser et al., 1997), further suggesting vulnerability to this compound.

![Figure 6: Killing of *B. burgdorferi* by mitomycin C (MMC).](image)

**Figure 6: Killing of *B. burgdorferi* by mitomycin C (MMC).** (a, c) Time-dependent killing of *B. burgdorferi* either at late exponential phase (a) or stationary phase (c) of growth were treated with MMC: 0.8 µg/ml (4X MIC) or 1.6 µg/ml (8X MIC). (b) Dose-dependent killing of late exponential cultures of *B. burgdorferi* culture after 5-day exposure to increasing concentrations of MMC. An aliquot was taken at indicated time points, washed, diluted, and plated on semi-solid BSK-II media for cfu counts. N=6. The x-axis is the limit of detection. An asterisk represents eradication to the limit of detection.

Mitomycin C eradicated a late exponential culture of *B. burgdorferi* within 24 hours, with no detectable persisters remaining (Figure 6a). This was observed with a low, clinically achievable dose of the compound - 1.6 µg/ml, or 8X MIC. In a dose-dependent experiment, eradication of a late exponential culture was achieved within 5 days with a 0.8 µg/ml (4X MIC) dose of the compounds (Figure 6b). Finally,
mitomycin C was tested against a stationary culture of *B. burgdorferi*. Surprisingly, eradication was achieved with a low dose of 4X MIC within 24 hours (Figure 6c). It appears that a stationary population is more susceptible to this compound than an exponentially growing one.

**Pulse-dosing.** Apart from identifying compounds capable of killing persisters, it may also be possible to eliminate them with conventional bactericidal antibiotics using pulse-dosing. Based on our results, the level of persisters is lowest during early exponential growth (Figure 2). We reasoned that allowing growth to resume and then re-treating them as they enter exponential phase could kill persisters surviving an antibiotic challenge. Eradication of the culture could then be achieved after several rounds of killing and regrowth. To test this, a culture of *B. burgdorferi* was exposed to amoxicillin or ceftriaxone. The surviving persisters were allowed to resuscitate for a short period of time in fresh media, and then exposed to antibiotic again for a second round of killing. Persisters were substantially diminished after four rounds of killing with amoxicillin, and were eradicated below the limit of detection after four rounds of killing with ceftriaxone (Figure 7). Additionally, we found that a ceftriaxone solution stored under experimental conditions (in BSK-II media at 34°C, 3% O₂, 5% CO₂) does not lose activity, as measured by MIC against *B. burgdorferi*, for up to 20 days. The activity of amoxicillin measured similarly, however, dropped 20-fold over 20 days which suggests degradation over time. The resulting MIC was still lower than the concentration used in killing experiments. This pulse-dosing experiment shows that a population of the pathogen can be eradicated with conventional antibiotics commonly used to treat the disease.
DISCUSSION

The presence of drug-tolerant persisters can explain the recalcitrance of chronic infections to antimicrobial therapy, especially in cases when the disease is caused by a susceptible pathogen. While some chronic infections are ancient – leprosy, syphilis, tuberculosis – many cases in developed countries are consequences of otherwise successful medical intervention. Various indwelling devices (catheters, prostheses, heart valves) provide a substratum for biofilms that protect persisters from the immune system (Lewis, 2010). Even in bacterial infections that are routinely successfully treated with antibiotics, there is dependence upon the host immune system to control persisting bacteria that are not eradicated by antibiotics. The role of the immune system becomes evident when these same infections involve immunocompromised hosts and antibiotic eradication of the infection becomes much more difficult.

*B. burgdorferi* is a pathogen that can affect immunocompetent hosts. It establishes long term infections of years to lifelong in both its natural (i.e. mice) and incidental (i.e. humans) hosts in the absence of antibiotic therapy (Barthold et al., 1993; Steere et al., 1987). Treatment in the early stages of
disease results in good outcomes. Delays in diagnosis and treatment lead to sequelae that may require additional treatment. For example, patients who develop arthritis, which typically begins after one month of untreated infection, often do not respond fully to a first course of 28 days of antibiotics (Marques, 2008). The majority of these patients have evidence of *B. burgdorferi* DNA in their synovial fluid and will respond to additional one or two month courses of antibiotics (Puius and Kalish, 2008; Wormser et al., 2006). A smaller minority of patients referred to as “antibiotic resistant Lyme arthritis” will continue to have arthritis with synovial fluid that is PCR negative for *B. burgdorferi* DNA. These patients typically respond to anti-inflammatory agents such as methotrexate or TNF-inhibitors. Both these groups of patients should be distinguished from the highly controversial group of patients with “chronic Lyme disease” that exhibit fatigue, myalgias and arthralgias without objective evidence of disease. For the first group of Lyme arthritis patients responsive to antibiotics, given that there is no reported resistance to clinically used tetracyclines, β-lactams, and cephalosporins in the pathogen, the need for lengthy courses of therapy is unclear. The presence of persister cells is one possible explanation and this is a pattern that is seen in other infections where persister cells are thought to be relevant for disease *in vivo*.

We found that similar to other pathogens, the pattern of killing of *B. burgdorferi* by bactericidal antibiotics is biphasic, with a small subpopulation of surviving persisters. These surviving clones are not resistant mutants; upon regrowth they form a new persister subpopulation. Also similar to *E. coli*, *S. aureus*, and other pathogens, the density of persisters increases as the culture deviates from strictly exponential growth, reaching a maximum at stationary state. This is probably due to a deterioration of growth conditions resulting in increasing numbers of dormant cells. However, of note, the stationary state in *B. burgdorferi* is atypical, as amoxicillin and ceftriaxone continue to kill the majority of cells despite an increase in the level of persisters in the population. Cell-wall acting antibiotics do not normally kill non-growing cells; one possibility is that stationary state *B. burgdorferi* cultures represent a steady state of growing and dying cells. The ability of β-lactams to kill non-growing cells has also been observed in *M. tuberculosis* where a combination of meropenem and a β lactamase inhibitor was able to kill viable but non-replicative cells (Hugonnet et al., 2009). The authors speculate that peptidoglycan remodeling continues in these non-replicating cells allowing for the activity of the β-lactam. This is another possible explanation of the killing we observe of stationary phase *B. burgdorferi* with amoxicillin and ceftriaxone.
In a recently published study, Iyer, et al. (Iyer et al., 2013) treated two different strains of \textit{B. burgdorferi} with ceftriaxone and were unable to detect live \textit{B. burgdorferi} by subculture in liquid medium. However, the cell density in that study was $10^7$ cells/ml, and according to our data, persister levels in this early exponential culture are low. In some of the biological replicates treated with ceftriaxone, we have not been able to recover live cells. At higher cell densities, the presence of persisters is unambiguous.

One common strategy for improving elimination of infective agents is to combine existing compounds. For example, β-lactams and aminoglycosides are known to synergize with each other to achieve effective killing of \textit{Enterococci} (Graham and Gould, 2002). We tested combinations of standard antibiotics used in treatment of Lyme disease as well as a combination of a fluoroquinolone and an aminoglycoside, compounds that often synergize and are capable of killing non-growing cells. However, there was no synergy in killing \textit{B. burgdorferi} with any of the tested combinations.

We recently described efficient killing of persisters in \textit{S. aureus} (Conlon et al., 2013) and in \textit{E. coli} (Fleck et al., 2014), and tested these compounds against \textit{B. burgdorferi}. ADEP4, an activator of the Clp protease, causes massive protein degradation in \textit{S. aureus}, killing regular cells and persisters. However, ADEP4 was not active against \textit{B. burgdorferi}. We also reported that nitrofuran prodrugs are effective in killing \textit{E. coli} persisters. Nitrofurans are reduced by bacterial nitroreductases into generally reactive compounds, explaining their activity against persisters. Nitroreductases are expressed under anaerobic or microaerophilic conditions. \textit{B. burgdorferi} is a microaerophilic organism, but does not have obvious homologs of a nitroreductase, and nitrofurans we tested were fairly inactive.

We also tested daptomycin, a lipopeptide that acts by increasing K⁺ permeability of the membrane. Being in a low-energy (microaerophilic) environment, the pathogen may be vulnerable to membrane-acting compounds. Daptomycin killed the majority of cells in a stationary culture, but the level of surviving persisters was comparable to that of a stationary culture treated with ceftriaxone. In a recent publication daptomycin was reported to kill \textit{B. burgdorferi} persisters more effectively than regular cells (Feng et al., 2014). This conclusion was based on equating stationary cells with persisters. As follows from our experiments, a stationary culture harbors a small subpopulation of persisters. The actual level of stationary cells apparently surviving treatment by daptomycin in that study was very high, 28%, as determined by live/dead staining. Under similar conditions, we detect about $10^3$ (0.002%) surviving
persisters by cfu count. It appears that live/dead staining may be over reporting the level of live *B. burgdorferi* cells.

Another weakness of the pathogen is its apparently limited ability for DNA repair. Based on the genome, *B. burgdorferi* lacks recFOR. In *E. coli*, both RecBC and RecFOR are required for repair of DNA damage caused by mitomycin C, an anticancer drug. Mitomycin C at a low, clinically achievable dose (8X MIC), eradicated *B. burgdorferi* persisters in both exponential and stationary cultures within 24 hours. A highly reduced environment activates mitomycin C, and this contributes to its selective action in microaerophilic tumors. While the killing of persisters by mitomycin C is impressive, given the toxicity of this drug, this is more of a proof-of-principle for a compound exploiting the weaknesses of this pathogen rather than a clinically useful agent. Treatment with mitomycin C can result in serious negative side effects and it should not be used for treatment of Lyme Disease. This agent will be useful to examine the possible contribution of persisters to the disease in an animal model of infection.

Another peculiar feature of *B. burgdorferi* and a weakness of the pathogen is the lack of development of resistance to any antibiotic used to treat Lyme disease. Even attempts to raise mutants resistant to amoxicillin and ceftriaxone *in vitro* have been unsuccessful. Joseph Bigger proposed an interesting strategy for elimination of persisters in 1944, in the first publication describing these cells (Bigger, 1944). The rationale is to add antibiotic to kill off regular cells; wash it away; allow the culture to start regrowing, at which point persisters will resuscitate. Reintroducing antibiotics will kill the regrowing bacteria. The argument against pulse dosing is that this protocol invites resistance development. Given that this is not a concern for *B. burgdorferi*, pulse dosing may be an effective strategy and we performed pulse dosing with amoxicillin and ceftriaxone. Persisters were eradicated with ceftriaxone in four pulses. These experiments form the basis for testing pulse dosing in an animal model, and if successful, in humans.

While we have identified the presence of *B. burgdorferi* persisters in cultures of the organism, the mechanisms by which they are able to survive remain unknown. There are multiple pathways of persister formation in other bacteria. The study of persisters so far identified redundant TA modules as a main component responsible for persister formation in *E. coli* and *S. typhimurium* (Dorr et al., 2010; Helaine et al., 2014; Maisonneuve et al., 2011). TA modules are widely spread among bacteria, but are surprisingly
absent from the genome of *B. burgdorferi*. Other components leading to persister formation in *E. coli* have been detected as well - the stringent response (Maisonneuve et al., 2013), various metabolic processes (Hansen et al., 2008; Spoering et al., 2006), global regulators, and protein stabilizing chaperones (Hansen et al., 2008). Future work will determine if these or other processes are involved in persister formation in *B. burgdorferi* and if persisters play a role in the pathogenesis of Lyme disease in humans.

**Acknowledgements:** This work was supported by grants from Lyme Research Alliance and by a T-R01AI085585 grant from the NIH to Dr. Kim Lewis and by grants R21AI082436 and U01AI109656 from the NIH to Dr. Linden T. Hu.

We thank Dr. Monica E. Embers from Tulane University for providing us with *B. burgdorferi* B31 5A19 strain and Dr. Yi-Pin Lin from Dr. John Leong’s lab at Tufts University for his help with the semi-solid plating method.

**Contributions:** BS & AVB conceived and performed experiments and wrote the chapter. NEM performed experiments. LTH & KL conceived experiments, provided expertise and feedback, and helped to write the manuscript.

**REFERENCES CITED**


Tuomanen, E., Cozens, R., Tosch, W., Zak, O., and Tomasz, A. (1986). The rate of killing of *Escherichia coli* by beta-lactam antibiotics is strictly proportional to the rate of bacterial growth. J Gen Microbiol 132, 1297-1304.


CHAPTER 3: PERSISTER FORMATION THROUGH ATP DEPLETION AND DECREASED TRANSLATION IN STAPHYLOCCUS AUREUS

Authors: Autumn Brown Gandt, Austin Nuxoll, Sarah Rowe, Brian Conlon, and Kim Lewis

ABSTRACT

*Staphylococcus aureus* is an important human pathogen that forms hard to treat biofilm infections, such as osteomyelitis and endocarditis. Persister cells, drug tolerant variants of a bacterial population, contribute to the recalcitrance of biofilm infections. *S. aureus* is unique in its complete tolerance to multiple classes of antibiotics during stationary phase. Due to the high persister levels in stationary phase, we reasoned that stresses and other factors that are present in stationary phase may trigger antibiotic tolerance. Based on this hypothesis, we investigated persister formation in *S. aureus* using two approaches. First, we studied mutants of likely persister genes, the two component systems which sense and respond to external and internal stimuli. Second, we subjected exponential cultures to stresses that are present during stationary phase. We found that the two component systems are not involved in persister formation. However, stresses that lower energy or decrease translation increase persister formation.

INTRODUCTION

*Staphylococcus aureus* is an important human pathogen. While it exists commensally in the nares of 20% of the population (Kluymans et al., 1997), *S. aureus* can cause disease in healthy and immunocompromised people and results in approximately 500,000 hospitalizations and 10,000 deaths per year in the United States (Klein et al., 2007). *S. aureus* forms antibiotic recalcitrant biofilm infections such as endocarditis, osteomyelitis, and biofilms on indwelling devices (Archer et al., 2011; Boucher et al., 2010; Conlon, 2014). Biofilms serve as a reservoir for antibiotic tolerant persister cells. Biofilms protect cells from many components of the immune system (Jesaitis et al., 2003; Leid et al., 2002; Vuong et al., 2004). Antibiotics can penetrate biofilms to kill normal cells but persisters survive to repopulate the infection after antibiotic treatment is completed. While resistance is a major problem in *S. aureus*, many
antibiotic failures are due to non-resistant strains (Conlon, 2014). Antibiotic tolerant persisters have been linked to chronic and recurring infections (LaFleur et al., 2010; Mulcahy et al., 2010; Schumacher et al., 2015). Furthermore, persisters can serve as a reservoir for the development of resistance (Lewis, 2007).

Molecular mechanisms of persister formation in *S. aureus* have not been extensively studied. Persister levels depend on the strain, antibiotic class, antibiotic concentration, and growth phase (Keren et al., 2004; Lechner et al., 2012). Persister levels are highly variable between biological replicates across multiple classes of antibiotics (Johnson and Levin, 2013). Toxin-antitoxin systems and the stringent response, which have been linked to persister formation in *E. coli* and *Salmonella* (Helaine et al., 2014; Maisonneuve et al., 2013), are not involved in persister formation in *S. aureus* (Conlon et al., 2016). One theory posits that persister formation in *S. aureus* is a result of the accumulation of random errors which slow growth or metabolism (Johnson and Levin, 2013). The theory is based on results showing that pretreatment of a culture with sub-inhibitory concentrations of one antibiotic increases survival to a different antibiotic. The authors conclude that stressors which cause cell damage increase persister formation.

Recently, a screen of a transposon mutant library for persister genes in *S. aureus* was performed (Wang et al., 2015). The group found that interruption of genes involved in the TCA cycle and electron transport chain decreased persister formation. These results suggest that energy generation is involved in persister formation in *S. aureus*.

Exponential cultures of *S. aureus* show traditional killing kinetics after antibiotic challenge, with a subpopulation of surviving persister cells. However, stationary phase cultures of *S. aureus* are completely tolerant to multiple classes of antibiotics, including beta lactams, the glycopeptide vancomycin, fluoroquinolones, and the RNA synthesis inhibitor rifampicin (Conlon et al., 2013; Keren et al., 2004; Lechner et al., 2012). The high tolerance of stationary phase cultures makes persister formation in *S. aureus* unique.

Because persister formation is "turned on" as cells transition from mid-exponential to stationary phase, we reasoned that cellular stresses present in stationary phase and systems that sense these stresses may be responsible for persister formation in *S. aureus*. We utilized two approaches to study
persister formation in *S. aureus*: screening genetic mutants of likely persister genes and subjecting exponential cultures to stresses designed to emulate stationary phase conditions.

**MATERIAL AND METHODS**

**Bacterial strains and growth conditions:** Bacteria were routinely cultured in Mueller Hinton broth at 37°C and 220RPM. Strains were either in the MRSA background JE2 or the MSSA background HG003.

**Two component system (TCS) screen:**

**Strains:** TCS mutant strains were transposon insertional mutants from the NARSA ordered library (Fey et al., 2013).

**Persistor assay:** *Gentamicin, tobramycin, and ciprofloxacin:* Overnight cultures were diluted 1:100 into fresh media. After two hours of growth, ciprofloxacin (160μg/mL), gentamicin (10μg/mL), or tobramycin (5 μg/mL) was added to each culture. An aliquot was diluted and plated before the addition of antibiotic and washed, diluted, and plated after 24 hours of treatment. Percent survival was calculated by dividing final cfu by initial cfu and multiplying by 100.

**Vancomycin:** Overnight cultures were diluted 1:1000 into fresh media. After two hours of growth, vancomycin (10μg/mL) was added to each culture. An aliquot was diluted and plated before the addition of antibiotic and washed, diluted, and plated after 24 hours of treatment. Percent survival was calculated by dividing final cfu by initial cfu and multiplying by 100.

**Stress persister assays:**

**Anaerobic condition:** Overnight cultures were diluted 1:100 into 30mL MH broth buffered with 100mM MOPS pH 7 in a 125mL baffled flask and incubated at 37°C, 220RPM for two hours. 15mL of culture was transferred to a 15mL conical, screw top tube. The lid was securely tightened to limit oxygen. Antibiotics were added to the remaining 15mL in the baffled flask 30 minutes later. After one hour, antibiotics as indicated were added to the low oxygen cultures in an anaerobic chamber. Cells were diluted and plated for cfu count before the addition of antibiotics. After a 24 hour challenge, an aliquot of each of the cultures was removed, washed with 1% NaCl, and plated for cfu. Percent survival was calculated by dividing final cfu by initial cfu and multiplying by 100.
Amino acid starvation: Overnight cultures were diluted 1:100 into MH broth. After three hours of growth, mupirocin (1μg/mL) was added. One hour later, cultures were challenged with ciprofloxacin (4μg/mL) or gentamicin (10 μg/mL). Cells were diluted and plated for cfu count before the addition of antibiotics. After a 24 hour challenge, an aliquot of each of the cultures was removed, washed with 1% NaCl, and plated for cfu. Percent survival was calculated by dividing final cfu by initial cfu and multiplying by 100.

Linezolid: Overnight cultures of HG003 were diluted 1:100 (cipro, gent) or 1:1000 (vanc) into MH broth and grown for three hours (cipro, gent) or two hours (vanc) before the addition of linezolid at the indicated concentration or challenge of the control cultures with cipro (4μg/mL), gent (10μg/mL), or vanc (10μg/mL). One hour after the addition of linezolid, antibiotics were added to the lin treated cultures at the same concentrations as the control cultures. Cells were diluted and plated for cfu count before the addition of antibiotics. After a 24 hour challenge, an aliquot of each of the cultures was removed, washed with 1% NaCl, and plated for cfu. Percent survival was calculated by dividing final cfu by initial cfu and multiplying by 100.

Arsenate: Overnight cultures of HG003 were diluted 1:100 into MH broth and grown for 2 hours. Arsenate was added at the indicated concentration. 40 minutes later, cipro (4μg/mL) or gent (10 μg/mL) was added to both the control and arsenate treated culture. Cells were diluted and plated for cfu count before the addition of antibiotics. After a 24 hour challenge, an aliquot of each of the cultures was removed, washed with 1% NaCl, and plated for cfu. Percent survival was calculated by dividing final cfu by initial cfu and multiplying by 100.

Translation and ATP assays:

Inducible GFP assay: HG003 pEPSA5 and pEPSA5::GFP overnight cultures were diluted 1:100 into fresh MH broth. Both stationary and diluted cultures were added to a 96 well plate at 200μL per well. The cultures were grown at 37°C, 220 RPM for 3 hours. Linezolid was added at indicated concentrations. One hour later, xylose (0.2%) was added to induce GFP expression from pEPSA5. Fluorescence (excitation 485, emission 528) and absorbance (OD600) were read every 30 minutes by a fluorimeter. GFP fluorescence was background subtracted using the empty plasmid and normalized by dividing by OD600.

ATP assay: Cultures were grown overnight in MH broth (stationary culture) or overnight cultures were diluted 1:100 into MH broth and grown for 3 hours (exponential culture). Arsenate was added to
exponential cultures at indicated concentration one hour before measuring ATP. ATP was measured using the BacTiter Glo Kit (Promega) according to the manufacturer’s instructions. Culture supernatant for each condition was used to background subtract extracellular ATP.

RESULTS

*S. aureus* has 14 two component systems (TCSs) which sense and respond to a variety of conditions, including oxygen levels (*srrAB, nreBC*), cell wall damage (*vraSR*), cell envelope stress (*nsaRS*), potassium or phosphate level (*kdpDE, phoRS*), and heme availability (*hssRS*) (Falord et al., 2011; Freeman et al., 2013; Hall and Ji, 2013; Kawada-Matsuo et al., 2013; Kinkel et al., 2013; Yang et al., 2013). TCSs are composed of a membrane bound histidine kinase and a cytoplasmic response regulator. In response to an external or internal stimuli, the histidine kinase phosphorylates and activates the response regulator which acts as a transcriptional regulator. Given their role in stress response, the TCSs seemed a logical start to search for persister genes in *S. aureus*.

The quorum sensing TCS *agrAC* was an especially attractive candidate for persister genes in *S. aureus* because both quorum sensing and persister formation are turned on at high cell density. In addition, quorum sensing has previously been linked to persister formation in *Pseudomonas aeruginosa* and *Streptococcus mutans* (Leung et al., 2015; Leung and Levesque, 2012; Que et al., 2013). The signaling molecule in the *agr* system is autoinducing peptide (AIP). AIP is encoded by *agrD*, modified and exported from the cell by AgrB, and activates the histidine kinase AgrC. Activated AgrC phosphorylates the response regulator AgrA, which activates transcription of the major regulatory RNA, RNAIII. RNAIII controls the transcription of several hundred genes, activation of the *agr* system results in upregulation of virulence factors, and down regulation of biofilm (Kong et al., 2006; Novick and Geisinger, 2008). There is a *luxS* gene in *S. aureus*, which is associated with quorum sensing in other species, however, it does not function in quorum sensing in *S. aureus* (Novick and Geisinger, 2008).

Mutants in the histidine kinase of all non-essential TCSs from the Nebraska ordered transposon mutant library of the MRSA strain JE2 were used (Fey et al., 2013). Each mutant was confirmed by PCR, assayed for minimum inhibitory concentration (MIC) to relevant antibiotics, and measured for growth rate (data not shown). If the histidine kinase mutant could not be confirmed by PCR, a mutant in the response
regulator was used instead. If the MIC or growth rate was different than the wild-type, the mutant was excluded from further analysis. The remaining TCS mutants were tested for survival during exponential growth after treatment with ciprofloxacin, an aminoglycoside (either gentamicin or tobramycin), and vancomycin (Figure 1). JE2 is resistant to ciprofloxacin, so a very high concentration (160μg/mL, 10X the MIC) was used. Gentamicin, tobramycin, and vancomycin were used at 10X the MIC. Due to the high variability of persister formation, only mutants with at least 10-fold difference in persister formation compared to the wild-type were considered to have a persister phenotype. Unexpectedly, none of the TCS mutants including \textit{agrC} had persister levels that were significantly different than the wild-type. I also assayed persister formation of an \textit{agrC} mutant in the MSSA background HG003 and an \textit{agrA} mutant in JE2, and found no impact on persister formation (data not shown).

![Figure 1](image1.png)

**Figure 1:** Two component systems do not contribute to persister formation in \textit{S. aureus}. TCS transposon mutants (gray bars) and the wild-type (white bars) were grown to exponential phase and challenged with (A) ciprofloxacin (Cipro, 160μg/mL), (B) vancomycin (Vanc, 10μg/mL), (C) gentamycin (Gent, 10μg/mL), or tobramycin (Tobra, 5 μg/mL). Percent survival was calculated by comparing the cfu prior to antibiotic challenge to the cfu after 24 hour antibiotic challenge. Data are the averages of at least three biological replicates. Error bars represent standard deviation.
I next considered whether stresses present in stationary phase trigger the formation of persister cells. In stationary phase oxygen levels are lower (Tolosa et al., 2002), nutrients are exhausted, translation rates slow, and energy levels are lower.

**Figure 2:** Transition to an anaerobic environment increases persister formation independently of nreC, srrB, and rex. HG003 wild-type (A) or indicated strains (B,C) were grown in a baffled flask to early exponential phase and half of the culture was removed and sealed tightly in a screw top tube (anaerobic). After 30 minutes (approximately one doubling), antibiotics were added to the aerobic cultures. One hour after being sealed in the tube (approximately one doubling), antibiotics were added to the anaerobic cultures in an anaerobic chamber. (A) Cultures were challenged with ciprofloxacin (Cipro, 4μg/mL), Gentamycin (Gent, 10μg/mL), or vancomycin (Vanc, 10μg/mL). (B) Cultures were challenged with gentamicin (10μg/mL). (C) Cultures were challenged with ciprofloxacin (4μg/mL). Percent survival was calculated by comparing the cfu prior to antibiotic challenge to the cfu after 24 hour antibiotic challenge. Data are the averages of at least three biological replicates. Error bars represent standard deviation.

Transitioning an exponential culture from a highly aerated environment to an oxygen limiting environment increased tolerance of the methicillin susceptible *S. aureus* (MSSA) strain HG003 to ciprofloxacin, gentamicin, and vancomycin (Figure 2A). This increase in persister formation was not dependent on the two TCSs srrAB and nreBC which sense and respond to low oxygen levels (Figure 2B).
(Hall and Ji, 2013). Similarly, a mutant in the master regulator rex, which controls the transition to anaerobic metabolism (Pagels et al., 2010), had persister levels similar to the wild-type under anaerobic conditions (Figure 2C). These results demonstrate that rex, nreBC, and srrAB are not responsible for the switch into the high persister state under anaerobic conditions.

Nutrients are exhausted in stationary phase. To emulate this condition in exponential phase, I utilized the antibiotic mupirocin. Mupirocin is an isoleucine analog which inhibits isoleucyl tRNA synthetase, mimicking amino acid starvation. Pretreatment of an exponential culture with mupirocin resulted in a 100-1000x increase in tolerance (Figure 3A). Translation is also slowed during stationary phase. Pre-treatment of an exponential culture with the translation inhibiting antibiotic linezolid at a concentration that stops translation (10μg/mL) increased tolerance to ciprofloxacin, gentamicin, and vancomycin (Figure 3B).

Energy in the form of ATP is lower in stationary than exponential cultures. ATP levels can be lowered using the phosphate analog arsenate. Arsenate competes with phosphate for addition to ADP by substrate level phosphorylation. ADP-arsenate is unstable and quickly hydrolyzes, resulting in a futile cycle which lowers ATP levels (Moore et al., 1983). Addition of arsenate at 15mM increased tolerance to both ciprofloxacin and gentamicin (Figure 3C).
I next wondered if the slowed translation and lowered energy present in stationary phase is sufficient to increase antibiotic tolerance. Using an inducible GFP system, translation capability was assayed during exponential growth and stationary phase (Figure 4A). Then, linezolid was used to titer down translation levels of an exponential culture. Linezolid at 2.5μg/mL decreases translation to approximately stationary levels (Figure 4A). An exponential culture was pre-treated with this concentration of linezolid and then challenged with ciprofloxacin or gentamicin. Decreasing translation rates to stationary levels increased antibiotic tolerance to gentamicin but not ciprofloxacin (Figure 4C). This suggests that slowing translation to the level that is present in stationary phase is not sufficient to confer tolerance to ciprofloxacin, even though stopping translation completely did result in increased tolerance (Figure 3B).
Similarly, relative ATP concentration was measured in exponential and stationary phase cultures using a luciferase based assay (Figure 4B). ATP was then lowered by adding increasing amounts of arsenate. Pre-treatment of an exponential culture with 5mM arsenate lowers ATP to a stationary phase level (Figure 4B). An exponential culture was pre-treated with this amount of arsenate and then challenged with ciprofloxacin or gentamicin. Lowering ATP to stationary-like levels resulted in increased tolerance to ciprofloxacin and to a greater extent gentamicin (Figure 4C). The fact that lowered ATP but not slowed translation increases tolerance to ciprofloxacin suggests that ATP concentration is more important than translation rate in ciprofloxacin tolerance.

Both slowed translation and lowered ATP increases tolerance to gentamicin. The effect is not additive, however, as a combination of linezolid and arsenate does not increase persister formation any more than arsenate alone (Figure 4C). There are two possible explanations, first that arsenate is preventing the uptake of gentamicin, which is energy dependent, resulting in tolerance. In this case, stopping translation will have no effect as there would be no gentamicin in the cell. The other possibility is that the concentration of arsenate used is sufficient to stop translation and the addition of linezolid has no further effect.
DISCUSSION

We found that the tested histidine kinases and response regulators of the two component systems, including the stringent response system \textit{agr}, do not contribute to persister formation under our conditions. It is possible that mutants in the response regulator rather than the histidine kinase, which were primarily what was assayed, could have had a persister phenotype. It is also possible that these systems may have a role to play in drug tolerance under conditions where they are activated. However,
srrAB and nreBC which are involved in the response to anaerobicity did not contribute to persister formation under anaerobic conditions.

We found that pretreatment with the antibiotic mupirocin, which mimics amino acid starvation, increases persister formation in *S. aureus*. Amino acid starvation has previously been linked to persister formation in *E. coli* (Maisonneuve et al., 2013). However, it has been shown that this increase in persister formation after treatment with the serine analog serine hydroxamate, an artificial way to induce amino acid starvation similar to mupirocin, is independent of the ppGpp mediated stringent response (See Chapter 4) (Amato et al., 2013). It is unclear from these experiments if the increase in tolerance in *S. aureus* after mupirocin treatment is due to the stringent response or stopped translation. It has been recently shown that ppGpp production is not involved in persister formation in *S. aureus* under normal growth conditions (Conlon et al., 2016). Future work will test whether an *rsh* mut mutant, which cannot produce ppGpp in response to amino acid starvation (Geiger et al., 2012), has increased persister levels after treatment with mupirocin.

Inhibiting translation has previously been linked to increased persister formation in *E. coli* (Kwan et al., 2013), but not in relation to the drop in translation seen in stationary phase. The question becomes, how does decreased translation increase persister formation? Lowered translation rates slow growth, increasing tolerance to the growth-rate dependent antibiotic vancomycin which targets cell wall synthesis. Stopping translation increases tolerance to aminoglycosides as they cause death by mistranslation. Translation-dependent killing by fluoroquinolones has previously been described (Drlica et al., 2008), though this phenomena is poorly understood. Fluoroquinolone tolerance after translation inhibition may be driven by decreased antibiotic targets; topoisomerase may be degraded and not re-translated. Another possibility is that transcription and replication are slowed when translation rates are low, resulting in less topoisomerase interacting with DNA.

As in *E. coli*, the evidence in *S. aureus* points to low energy, specifically low ATP, being a second cause of multidrug tolerant persister formation. Antibiotics kill by corrupting active targets: fluoroquinolones inhibit the re-ligation step of topoisomerase, turning it into an endonuclease; aminoglycosides target the ribosome and cause mistranslation; vancomycin inhibits peptidoglycan crosslinking during cellular growth, resulting in cell lysis (Kohanski et al., 2010). All of these are active
cellular processes that require ATP. Under low ATP conditions, the antibiotic targets are less active and more cells are multidrug tolerant persisters.

Lowered translation and ATP levels are a satisfactory explanation for antibiotic tolerance in stationary phase *S. aureus* cultures. What remains less clear is the nature of the drug-tolerant persister subpopulation present in exponential phase. A concurrent study performed in our lab found that the persisters present in exponential phase are cells that have entered a stationary-like state (Conlon et al., 2016). The genes *arcA* and *cap5A* are highly expressed in stationary phase, but not exponential phase. The promoters of these genes were used to drive *gfp* expression and these constructs were used as stationary phase reporters. Fluorescent-activated cell sorting (FACS) was used to sort cells with differing expression levels of the reporters from an antibiotic treated exponential culture. Cells with high expression of ParcA or Pcap5A were enriched for persisters compared to the bulk of the population. We conclude that these persisters are cells that have entered a stationary-like state before the rest of the population. Indeed, it was found that the *arcA* and *cap5A* reporters responded to low ATP levels: expression increased after arsenate treatment (Conlon et al., 2016). This suggests that persisters are low ATP, stationary-like cells.

**FUTURE WORK**

This project has established that artificially lowering intracellular ATP levels increases persister formation. It remains to be determined if low ATP cells exist naturally in the population and whether such cells are tolerant to antibiotics. We plan to optimize a FRET-based ATP biosensor (Maglica et al., 2015) for expression in *S. aureus*. Using this construct and fluorescent microscopy, we will identify low ATP cells in a population, treat the population with a lethal dose of an antibiotic, and observe whether the low ATP cells are able to survive the challenge to regrow after the removal of antibiotic. This experiment will provide further evidence that low ATP causes persister formation in an unperturbed population.

We also aim to more definitively show the link between low ATP and low antibiotic target activity. We plan to establish that arsenate treatment decreases translation (ribosome) activity, the target of aminoglycosides using an inducible GFP assay. We will perform additional assays to establish that transcription, the target of rifampicin, and double strand break formation, the mechanism of action of
fluoroquinolones, are lowered after arsenate treatment. Using established methods (Gavrieli et al., 1992), we will measure double strand break formation after ciprofloxacin treatment in control and arsenate treated cultures. We will utilize qRT-PCR to establish if transcription rates are lowered after arsenate treatment. These experiments will provide a causal link between lowered ATP and increased drug tolerance.

We will also search for the genetic basis for translation and ATP heterogeneity in *S. aureus* cultures. A screen of persister formation in the entire Nebraska transposon mutagenesis library has been performed to identify mutants with low persister formation. Translation capability and ATP levels of all hits will be compared to the wild-type in an attempt to find genes that modulate these important functions. It is possible that there is no genetic basis for ATP and translation heterogeneity, rather, stochastic mistakes in the cell lead to lowered energy and or translation capability.

**Contributions:** Kim Lewis, Brian Conlon, Sarah Rowe, and Austin Nuxoll provided helpful discussion and expertise. Kim Lewis and Brian Conlon provided a critical reading of this chapter.

**REFERENCES CITED**


CHAPTER 4: STOCHASTIC FORMATION OF DRUG-TOLERANT PERSISTERS IN ESCHERICHIA COLI

Authors: Autumn Brown Gandt*, Yue Shan*, Sarah E. Rowe, Julia P. Deisinger, Brian P. Conlon, and Kim Lewis

*Co-first authors

ABSTRACT
Persisters are dormant variants that form a subpopulation of drug-tolerant cells largely responsible for recalcitrance of chronic infections, and toxin-antitoxin (TA) modules have been linked to their formation in Escherichia coli. The current model holds that the stringent response upregulates ppGpp synthesis by RelA/SpoT, which causes activation of the Lon protease to degrade antitoxins; active toxins then inhibit translation, resulting in dormant, drug-tolerant persisters. We find that TA modules are upregulated in response to various stresses, including stringent response. Stringent response increased tolerance to ciprofloxacin in a TA dependent manner, but increased tolerance to ampicillin was not due to the action of the TAs. Unexpectedly, no other stress condition had a similar TA dependent increase in persister formation, despite upregulation of up to eight toxins. A relA/spoT mutant formed less persisters, but this was cell density dependent. Activity of the ribosomal rrnB P1 promoter has been used to detect persisters, and it reports both ppGpp and ATP levels. We find that persisters can be isolated by sorting cells highly expressing rrnB P1-GFP in the background of a relA/spoT mutation. Consistent with this finding, decreasing the level of ATP by treatment with arsenate causes drug tolerance. We conclude that stochastic variation in ATP levels is the main mechanism of multi-drug tolerant persister formation.

INTRODUCTION
Chronic infections are caused mainly by drug-susceptible pathogens, but are difficult to eradicate (Conlon, 2014). This is particularly true for biofilms, microbial communities that form on indwelling devices or within soft tissues and are protected from the immune system by a layer of exopolymers (Leid et al., 2005; Vuong et al., 2004). An increasing body of evidence points to the main culprit of drug tolerance, persister cells. Produced stochastically by all pathogens studied, persisters are dormant phenotypic
variants of the wild-type. Bactericidal drugs kill by corrupting active functions, which explains why dormant cells are tolerant to antibiotics (Keren et al., 2013).

Most of what we know about the mechanism of persister formation comes from the study of *E. coli*. Isolated persisters express interferases (Keren et al., 2004b; Shah et al., 2006), mRNA-degrading toxin-antitoxins (TA) (Gerdes et al., 2005). Ectopic expression of toxins causes multidrug tolerance (Keren et al., 2004b; Maisonneuve et al., 2011). Stochastic overexpression of the *yoeB* toxin in individual cells has been reported to protect from ampicillin as well (Maisonneuve et al., 2013b). Deleting single interferases does not produce a phenotype (Shan et al., 2015), but the level of persisters was reported to be drastically decreased in a strain deleted in 10 TA interferases (Δ10TA) (Maisonneuve et al., 2011). Stochastic expression of the *HipA* toxin which inhibits protein synthesis by phosphorylating gln-tRNA synthetase (Germain et al., 2013; Kaspy et al., 2013) also contributes to formation of persisters (Germain et al., 2013; Schumacher et al., 2015). Gain of function mutations in *hipA* produce elevated levels of persisters in vitro, and the same mutants are present in patients with relapsing urinary tract infection (UTI) (Schumacher et al., 2015). *E. coli* causing UTI forms drug tolerant biofilms within bladder epithelial cells (Anderson et al., 2003; Rosen et al., 2007). High persister (hip) mutants are also common among *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis (Mulcahy et al., 2010), and among *Candida albicans* from patients with oral thrush (LaFleur et al., 2010). These observations link persisters to clinical manifestation of chronic disease.

Apart from random variation in gene expression and hip mutations, stress has been linked to increase in drug tolerance. Fluoroquinolone antibiotics kill cells by forcing the DNA gyrase and topoisomerase to cleave double-stranded DNA, which induces the SOS response (Radman, 1975). The main function of SOS is to express DNA repair enzymes, but the same regulatory pathway also turns on production of the *TisB* toxin in a subpopulation of *E. coli* cells (Dorr et al., 2010). *TisB* forms an ion channel in the cytoplasmic membrane, decreasing the proton motive force and ATP, which leads to drug tolerance (Dorr et al., 2010). Stress in the form of starvation has also been linked to expression of TA interferases (Christensen et al., 2001). Specifically, formation of ppGpp by the stringent response has been reported to cause an increase in persisters (Amato et al., 2013; Nguyen et al., 2011). Induction of TA interferases by stringent response has become a widely accepted model for persister formation in *E.
coli (Gaca et al., 2015; Helaine et al., 2014; Helaine and Kugelberg, 2014; Holden, 2015; Maisonneuve et al., 2013a).

In this study, we show that different stresses induce TA interferase modules, but, unexpectedly, this does not lead to persister formation in most cases. Tolerance to ciprofloxacin under the stringent response is the only TA dependent increase in tolerance we identified. We find that a ppGpp₀ strain has a low persister phenotype under certain conditions but mutants of lon and ppx do not. A drop in ATP correlates with dormancy and accounts for drug tolerance of persisters.

MATERIALS AND METHODS

Bacterial strains and growth conditions

*E. coli* MG1655 and its derivatives were cultured in Luria Bertani (LB) broth or MOPS minimal medium (Neidhardt et al., 1974) supplemented with 0.2% glucose where indicated. Bacteria were routinely grown at 37°C at 220 RPM. Media was supplemented with kanamycin 25 µg/ml to maintain plasmids where necessary.

Deletion mutants were constructed using P1 transduction from the KEIO collection of *E. coli* deletion strains into MG1655 (Baba et al., 2006). The kanamycin cassette was cured using the Flp recombinase system on pCP20 (Cherepanov and Wackernagel, 1995).

The ΔrelAΔspoT deletion mutant was constructed by first transducing the relA deletion allele from the KEIO collection as described above into MG1655. A clean deletion of spoT was made using the red recombinase methods described in previous study and removing the antibiotic cassette with pCP20 (Datsenko and Wanner, 2000).

The rmB P1::gfp unstable strains were constructed by P1 transduction from the donor strain ASV.

All strains were confirmed by PCR.
### Table 1: Strain List

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655 (WT)</td>
<td></td>
</tr>
<tr>
<td>MG1655 pUA66 P&lt;sub&gt;chpSB&lt;/sub&gt;::gfp</td>
<td>This work</td>
</tr>
<tr>
<td>MG1655 pUA139 P&lt;sub&gt;dinJyafQ&lt;/sub&gt;::gfp</td>
<td>(Zaslaver et al., 2006)</td>
</tr>
<tr>
<td>MG1655 pUA66 P&lt;sub&gt;hicA&lt;/sub&gt;::gfp</td>
<td>This work</td>
</tr>
<tr>
<td>MG1655 pUA139 P&lt;sub&gt;yefMyoeB&lt;/sub&gt;::gfp</td>
<td>This work</td>
</tr>
<tr>
<td>ΔyafQ::frt</td>
<td>This work</td>
</tr>
<tr>
<td>Δ10TA</td>
<td>(Maisonneuve et al., 2011)</td>
</tr>
<tr>
<td>Δlon</td>
<td>(Maisonneuve et al., 2013b)</td>
</tr>
<tr>
<td>ΔlonΔsulA</td>
<td>(Maisonneuve et al., 2013b)</td>
</tr>
<tr>
<td>Δppx::frt</td>
<td>This work</td>
</tr>
<tr>
<td>Δppk::frt</td>
<td>This work</td>
</tr>
<tr>
<td>ΔrelA::frt ΔspoT::frt</td>
<td>This work</td>
</tr>
<tr>
<td>ΔyafQ::frt</td>
<td>This work</td>
</tr>
<tr>
<td>Δ10TA</td>
<td>This work</td>
</tr>
<tr>
<td>Δlon</td>
<td>This work</td>
</tr>
<tr>
<td>ΔlonΔsulA</td>
<td>This work</td>
</tr>
<tr>
<td>ΔrelA::frt Δppk::frt</td>
<td>This work</td>
</tr>
<tr>
<td>ΔrelA::frt Δppx::frt</td>
<td>This work</td>
</tr>
</tbody>
</table>

### Table 2: Primers for creation of plasmid based GFP promoter fusions (Zaslaver et al., 2006):

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUA139 P&lt;sub&gt;yefM&lt;/sub&gt;::GFP 1</td>
<td>GTAGTAGGATCCCAGGATGATGATGATGATGG</td>
</tr>
<tr>
<td>pUA139 P&lt;sub&gt;yefM&lt;/sub&gt;::GFP 2</td>
<td>GTAGTACTCGAGTTGGTGCTCGACAAATTCTGA</td>
</tr>
<tr>
<td>pUA66 P&lt;sub&gt;chpS&lt;/sub&gt;::GFP 1</td>
<td>GTAGTACTCGAGTTGAACCGCGCGACTG</td>
</tr>
<tr>
<td>pUA66 P&lt;sub&gt;chpS&lt;/sub&gt;::GFP 2</td>
<td>GTAGTAGGATCCGGGAATGACCATACCTGCAC</td>
</tr>
<tr>
<td>pUA66 P&lt;sub&gt;prlF&lt;/sub&gt;::GFP 1</td>
<td>GTAGTACTCGAGCCGATACCGACTG</td>
</tr>
<tr>
<td>pUA66 P&lt;sub&gt;prlF&lt;/sub&gt;::GFP 2</td>
<td>GTAGTAGGATCCCTACGACGCCCTTGAGAT</td>
</tr>
</tbody>
</table>

### Table 3: Primers for the deletion of spoT (Cherepanov and Wackernagel, 1995):

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>spoT del 1</td>
<td>TTGTATCTGTTTGAAAGCCTGAATCAACTGATTCAAACTACCTGACGCCGATTG</td>
</tr>
<tr>
<td></td>
<td>TAGGCTGGAGGTCCTGCGTCA</td>
</tr>
<tr>
<td>spoT del 2</td>
<td>TTAATTTCGTTTGGTGGTGAATTTAATCAGGTCGTGAATCAAATCAGGTCGTGAATCAAATCATACCTG</td>
</tr>
<tr>
<td></td>
<td>TATGATTATACGAGTATGAGAGAGATGAGAGATGAGAGATGAGAGATGAGAGATGAGAGATGAGAGAT</td>
</tr>
</tbody>
</table>
Stress induction, TA expression and survival measurement:
Bacterial strains were grown under stress conditions and compared to non-stress conditions; promoter activity and antibiotic survival was measured. Stress was induced as follows:

**Osmotic stress:** Cultures were grown to early exponential phase in MOPS minimal media. Water (control) or sucrose to 330 mM (stress) was added (Stoebel et al., 2009).

**Isoleucine starvation:** Cultures were grown in MOPS medium supplemented with all 20 amino acids with a final concentration of 400 μM isoleucine (control) (Wanner et al., 1977) or isoleucine concentration was reduced to 60μM (stress) (Traxler et al., 2008). An additional 100mM MOPS adjusted to pH 7 was added to the media to prevent pH change.

**Acid stress:** Cultures were grown in either unbuffered LB medium (control) or with the addition of 100mM MES adjusted to pH 5 (stress). Ciprofloxacin is sensitive to low pH, so ciprofloxacin tolerance was not assayed under acid stress.

**Phosphate starvation:** Cultures were grown in MOPS minimal medium with 1.32mM KH₂PO₄ (control), which is 10-fold more than the phosphate limiting media (stress) (Luttmann et al., 2012).

**Sodium stress:** Cultures were grown in MOPS minimal medium to early exponential phase, then an additional 300mM NaCl was added (stress). Control cultures had no additional NaCl (Stoebel et al., 2009).

**Promoter induction measurement:** MG1655 harboring *gfp* promoter plasmids from the *E. coli* promoter library were used for TA stress induction measurements (Zaslaver et al., 2006). When an interferase promoter was not present in the library, a plasmid was constructed using the same method as described (Zaslaver et al., 2006). All plasmids were confirmed by sequencing. The *gfp*-promoter strains were grown in 96 well plates in a fluorimeter at 37° C under the conditions described above. Media was supplemented with kanamycin (25 μg/ml) to maintain plasmids. Absorbance at OD600 and GFP fluorescence (emission 528nm, excitation 485nm) were measured every 30 minutes. The GFP value was background subtracted using a strain carrying a plasmid expressing promoterless *gfp*. To correct for the effect of slowed growth by stresses, OD versus GFP values were plotted. Each GFP fluorescence value that fell within 0.1 OD unit bin for stress and non-stress conditions were compared. The average of all "stress" GFP values were
divided by the average of all “non-stress” GFP values. It was determined that a toxin had increased expression under a stress if there was a statistically significant (p<0.05 by Student’s t test) increase of GFP fluorescence of at least 50% during three independent experiments. The analysis was limited to early exponential phase (OD<0.5), to correspond to when antibiotics were added in the antibiotic survival assays.

**Antibiotic survival assay:** Bacteria were inoculated at 1:100 into LB medium or 1:50 into MOPS-based media from an overnight culture. Cell cultures were grown for 2 h- 3.5 h to reach approximately the same cfu (1-4 x 10^8 cfu/ml). Ampicillin (100 µg/ml) or ciprofloxacin (concentration as indicated) was added. At each time point after addition of antibiotic, cultures were washed with 1% NaCl and plated on LB agar for cfu count. The percent survival was calculated as (final cfu / cfu at 0h) x 100. Results are averages from at least 6 biological replicates, and error bars represent standard deviation. P values were calculated by a two tailed Student’s t test and a P value less than 0.05 is considered significant.

In the ars enate, chloramphenicol, and SHX antibiotic survival assays, methods were followed as above, except cultures were pre-treated with arsenate (30min), chloramphenicol (45min), or SHX (30min) before the addition of antibiotics.

**Flow cytometry analysis and cell sorting:** Fluorescent protein level was analyzed with a BD Aria II flow cytometer (BD Biosciences) with a 70-micron nozzle. Cell population was detected using forward and side scatter parameters (FSC and SSC), and fluorescence was analyzed with emitting laser of 488 nm and bandpass filter of 525/15 nm. For each sorting experiment, 32000 events were collected from each population and dispensed directly to agar plate. The survival rate was calculated as cfu divided by total cells sorted to plate. Results of survival are averages from at least 6 biological repeats, and error bars represent standard deviation. P values were calculated by the Student t test and P value smaller than 0.05 is considered significant. Data were acquired using FACSDiVa software and graphs were generated by FlowJo (Tree star software).

**ATP measurement:** ATP levels of stationary and exponential cultures with the addition of various concentrations of arsenate were measured using BacTiter Glo kit (Promega) according to the manufacturer’s instructions. Background ATP was subtracted using spent media from each condition.
RESULTS

TA interferases are upregulated by stress

Some interferase TAs are expressed during starvation induced by serine hydroxamate or glucose limitation (Christensen-Dalsgaard et al., 2010; Christensen et al., 2001; Christensen et al., 2003; Jorgensen et al., 2009), and it was suggested that stress induces persister formation (Poole, 2012). However, evidence linking induction of interferases during stress and persister production is lacking. We examined this relationship, and screened 10 TA modules for induction of transcription by several stresses – acid, high sodium, high osmolarity, isoleucine starvation (stringent response), and phosphate starvation. *E. coli* strains from a library of promoter-*gfp* fusions (Zaslaver et al., 2006) were used for this purpose. All of the TAs were induced by at least one stress (Table 1, Figure 1). Isoleucine starvation induced 10 TAs, followed by osmotic stress (8), acid stress (6), phosphate stress (6), and NaCl stress (1).
Increased expression of the TA interferases caused by isoleucine starvation agrees with previously published data describing induction of many of these TA systems by isoleucine starvation and serine hydroxamate (SHX), an artificial activator of stringent response (Christensen-Dalsgaard et al., 2010; Christensen et al., 2001; Christensen et al., 2003; Jorgensen et al., 2009; Traxler et al., 2008).

Next, we tested whether stresses induce persister formation. Isoleucine starvation and NaCl stress increased the level of persisters surviving treatment with ampicillin or ciprofloxacin 10-100 fold.
(Figure 2B,E). Phosphate starvation, acid stress, or osmotic stress had no effect on drug tolerance in spite of strong induction of up to eight TAs (Figure 2).

We next tested whether increased persister formation due to isoleucine starvation or NaCl stress was dependent on TA modules. For this, we deleted yafQ which is upregulated upon NaCl stress and utilized the Δ10TA strain with 10 interferase TA systems deleted because we found all ten interferases to be upregulated in response to isoleucine starvation (Maisonneuve et al., 2011). Persister levels of the yafQ knockout strain was the same as the wild-type when exposed to NaCl stress (Figure 2G). Unexpectedly, the wild-type and Δ10TA strain had a similar increased tolerance to ampicillin under starvation conditions (Figure 2F). However, ciprofloxacin tolerance increased in the wild-type and not the Δ10TA strain under starvation (Figure 2F). This indicates that the increased tolerance to ciprofloxacin, but not ampicillin, under isoleucine starvation is driven by the action of the toxin-antitoxin interferases.

The Δ10TA strain is reported to have low persister levels after challenge with ampicillin or ciprofloxacin (Maisonneuve et al., 2011). Unexpectedly, we found that the low persister phenotype of the Δ10TA strain is antibiotic specific under these conditions: it has lower tolerance than the wild-type to ciprofloxacin, but not ampicillin. We decided to explore this further.
Figure 2: Upregulated expression of TA modules induces persister formation only under isoleucine starvation.

(A-E) MG1655 (WT) was grown under stress as described in materials and methods to approximately the same starting density as the control. Cultures were then challenged with ciprofloxacin (0.5 μg/ml) or ampicillin (100 μg/ml) for 4 (A, C-E) or 5 (B) hours.

(F, G) Strains deleted in toxins showing increased expression under sodium stress or isoleucine starvation were tested for persister formation. The Δ4 strain is deleted in chpB, higB, mazF, and relE. MG1655 (WT) and isogenic mutant strains were grown to the same cell density under the indicated stress and then challenged with ampicillin (100 μg/ml) or ciprofloxacin (0.5 μg/ml) for 5 hours (F) or 4 hours (G).

Results are expressed as % survival by comparison to untreated culture prior to the addition of antibiotic. Data are averages of at least two independent experiments performed with three biological replicates (n≥6). A * indicates a significant difference (P<0.05) by a two tailed Student’s t test. Error bars represent standard deviation.
Δ10TA strain’s multi-drug low persister phenotype depends on the media used

The Δ10TA strain is reported to have a low tolerance to both ampicillin and ciprofloxacin when grown in rich medium (Maisonneuve et al., 2011). Indeed, we find that Δ10TA has a lower level of persisters surviving treatment with ciprofloxacin and ampicillin when grown in LB (Figure 3AB), in general agreement with previous studies (Maisonneuve et al., 2011). However, the phenotype we observe with both antibiotics is more modest than previously reported. With ampicillin, the difference in persister levels between wild-type and Δ10TA is 30 fold (vs. reported 200 fold); and 16 fold vs. 200 fold for ciprofloxacin. The low persister phenotype after ampicillin challenge virtually disappeared when the strains were grown in MOPS minimal medium (Neidhardt et al., 1974), however, with the Δ10TA strain having a small but statistically significant 2-fold lower number of persisters than the wild-type (Figure 3C). In MOPS medium, the Δ10TA strain maintains a low persister phenotype when challenged with ciprofloxacin; Δ10TA was killed to below the level of detection (100cfu/mL) in 4 out of 5 replicates by ciprofloxacin while the wild-type’s persister level is at least 8-fold higher (Figure 3D). In a chemically defined medium with all 20 amino acids added (“MOPS + AA”, the control medium for isoleucine starvation experiments) (Traxler et al., 2008), again, the Δ10TA strain has a low persister phenotype when challenged with ciprofloxacin but not ampicillin (Figure 3E,F). It seems that the low persister phenotype of the Δ10TA strain is specific to ciprofloxacin; the phenotype with ampicillin is only present in rich media.
Figure 3: Low tolerance of Δ10TA is dependent on antibiotic and growth conditions

(A,B) Cultures were grown to exponential phase in LB medium. MG1655 (WT) and the isogenic interferase deletion strain (Δ10TA) were exposed to either ampicillin (amp, 100 µg/ml) (A) or ciprofloxacin (cip, 1 µg/ml) (B).

(C,D) Cultures were grown to exponential phase in MOPS minimal medium. MG1655 (WT) and the isogenic interferase deletion strain (Δ10TA) were exposed to either ampicillin (amp, 100 µg/ml) (C) or ciprofloxacin (cip, 0.5 µg/ml) (D).

At time 0, an aliquot of a culture was diluted and plated for colony forming units (cfu). At each subsequent time point, an aliquot of the culture was washed and plated to quantify persisters. Data are averages of at least two independent experiments performed with two-three biological replicates (n≥5). Error bars represent standard deviation.
Δ10TA decreases persister level through a translation independent mechanism

mRNA interferases were linked to persister formation as ectopic overexpression of these genes leads to rapid degradation of mRNA and shutdown of translation (Gerdes et al., 2005). It has been proposed that stochastic activation of toxin expression similarly inhibits translation, leading to persister formation (Germain et al., 2015; Maisonneuve et al., 2013b). Failure to inhibit translation is then the reason for the lower persister levels in Δ10TA. We reasoned that if interferases act by inhibiting protein synthesis, Δ10TA should have the same level of persisters as the wild-type in the presence of an inhibitor of translation. To test this, we pretreated cells with chloramphenicol to shutdown translation. At high concentration, chloramphenicol stops protein synthesis, as measured by expression of inducible *gfp* (Figure 4A). As expected, inhibition of translation increases persister formation in the wild-type (Figure 4B). However, Δ10TA still forms fewer persisters tolerant to ciprofloxacin as compared to the wild-type in the presence of chloramphenicol (Figure 3). These results indicate that deletion of 10 TAs decreases the level of persisters tolerant to fluoroquinolones through a translation independent mechanism.

**Figure 4:** Induction of persisters by toxins does not depend on inhibition of translation.

(A) MG1655 wt harboring a plasmid-borne *P_{lacZ}::GFP* fusion was grown to exponential phase. Cultures were pretreated with chloramphenicol (100μg/ml) (cam) for 30 minutes where indicated. IPTG (1mM) was then added to induce expression of GFP from the *lacZ* promoter. GFP fluorescence (excitation 485, emission 528) was measured every 30 minutes. Data points are the average of the experiment performed in triplicate (n=3). Error bars (too small to see) represent standard deviation.

(B) MG1655 (WT) and isogenic TA deletion strain (Δ10TA) were grown to exponential phase in LB medium and exposed to 1 μg/ml ciprofloxacin (left panel). For inhibition of translation, cultures were incubated with 100 μg/ml chloramphenicol for 45 minutes before antibiotic exposure (right panel). Results are expressed as % survival by comparison to untreated culture prior to the addition of antibiotic. Data are averages of at least two independent experiments performed with three biological replicates (n≥6). Error bars represent standard deviation.
Lon and PolyP are not involved in persister formation

In light of the results described above, we decided to systematically re-examine the current model of persister formation which is widely accepted (Gaca et al., 2015; Helaine et al., 2014; Helaine and Kugelberg, 2014; Maisonneuve et al., 2013b). According to this model, starvation induces ppGpp synthesis by RelA/SpoT, the alarmone inhibits the PPX phosphatase, and increased levels of polyphosphate activate the Lon protease that degrades the antitoxins, active toxins are released, inhibiting translation which causes drug tolerance.

Persister levels are diminished in lon mutants exposed to ciprofloxacin (Theodore et al., 2013). This is to be expected, since ciprofloxacin damages DNA, which induces the SOS response and the synthesis of SulA, an inhibitor of cell division. When DNA is repaired, SulA is degraded by Lon. In a lon mutant SulA accumulates, leading to cell elongation and eventual lysis and death (Schoemaker et al., 1984). According to our findings, a lon sulA double deletion mutant has no persister phenotype when challenged with ciprofloxacin (Theodore et al., 2013). This is in disagreement with Maisonneuve and co-authors, who reported a low persister phenotype even in a lon sulA mutant (Maisonneuve et al., 2013b). We found that Δlon has a low persister phenotype when challenged with ciprofloxacin, but as we had previously shown, the ΔlonΔsulA mutant has persister levels similar to those of the wild-type (Figure 5B). We found no difference in persister levels in either the Δlon or ΔlonΔsulA strains when they were challenged with ampicillin (Figure 5A). Thus, Lon does not appear to affect persister formation.
Maisonneuve and co-authors reported that a strain deleted in PolyP hydrolase *ppx* has increased persister levels (Maisonneuve et al., 2013b). We find no difference in persister levels between the *ppk* or *ppx* mutants and the wild-type (Figure 5C). This is in agreement with a recent report from Verstraert et al. who similarly did not find an altered persister phenotype in *ppk* and *ppx* mutants (Verstraert et al., 2015). These results agree with a lack of an effect of Lon on tolerance. We conclude that activation of Lon by PolyP does not play a role in persister formation.

The involvement of stringent response in persister formation has been implicated by several independent groups (Amato et al., 2013; Korch et al., 2003; Maisonneuve et al., 2013b; Nguyen et al.,...
The principal observation was that persister levels are decreased in a \textit{relA/spoT} mutant and are increased by upon activation of stringent response. We constructed a \textit{ΔrelAΔspoT} double deletion mutant, which was confirmed by PCR as well as by its inability to grow in minimal media. The \textit{ΔrelAΔspoT} mutant has a growth defect and does not reach the same cell density as the wild-type in stationary phase (Figure 6A). When the wild-type and mutant strains were grown to early exponential phase (\~5\times10^7 cfu/mL), the \textit{ΔrelAΔspoT} mutant showed a low persister phenotype, in agreement with previous reports (Figure 6B,C). However if the strains were grown instead to a later growth phase (\~2\times10^8 cfu/mL), the \textit{ΔrelAΔspoT} strain had persister levels that are similar to the wild-type when challenged with ciprofloxacin, and showed a modestly decreased level of persisters tolerant to ampicillin (Figure 5D,E). The decrease in multi-drug tolerance of \textit{ΔrelAΔspoT} appears to be cell density dependent.
Serine hydroxamate (SHX) has been used previously as a tool for inducing the stringent response and testing its effect on persister formation (Amato et al., 2013). SHX is an analog of serine that inhibits serine tRNA synthetase, resulting in unoccupied codons and an induction of the stringent response. 

Figure 6: Effect of relA spoT mutation on persister formation

(A) Overnight cultures of MG1655 (WT) and an isogenic ΔrelAΔspoT mutant were diluted 1:100 into LB media and growth was monitored by plating for cfu. MG1655 (WT) and the ΔrelAΔspoT mutant were grown either to early exponential phase (B,C) or mid-exponential phase (D,E) and exposed to ampicillin (100 µg/ml) or ciprofloxacin (1 µg/ml) as indicated. At time 0, an aliquot was diluted and plated for initial cfu. At each subsequent time point, an aliquot of the culture was washed and plated to quantify persisters. Data are averages of at least two independent experiments performed with three biological replicates (n≥6). Error bars represent standard deviation.
response (Pizer and Merlie, 1973). We tested the effect of SHX on persister formation in the ΔrelAΔspoT mutant and found that it increases persister formation (Figure 7A) as reported previously (Amato et al., 2013). The effect of SHX on drug tolerance is therefore unrelated to its ability to induce the synthesis of ppGpp. SHX treatment likely stops translation and induces persister formation independently of the stringent response.

Isoleucine starvation is another established method to induce the stringent response in E. coli K12 (Traxler et al., 2008). Similarly to SHX treatment, isoleucine starvation increased persister formation both in the wild-type and the ΔrelAΔspoT mutant (Figure 7B). This shows that ppGpp is not required to increase persister formation under isoleucine starvation. This suggests that the low ciprofloxacin tolerance of the Δ10TA strain under isoleucine starvation is independent of ppGpp.

**rrnB P1 reports persister formation independently of TA interferases and ppGpp**

In a growing population, regular cells have high levels of expression of 16S RNA, controlled by the rrnB P1 promoter. Cells with an inactive rrnB P1 promoter are likely dormant, and sorting out dim cells
of a strain expressing degradable GFP under control of this promoter resulted in isolation of persisters (Shah et al., 2006). In an independent study, RpoS-mCherry was used as a marker for ppGpp to identify persisters (Maisonneuve et al., 2013b). ppGpp activates RpoS transcription and inhibits its proteolysis (Battesti et al., 2011). Bright RpoS-mCherry cells did not grow and were not killed by ampicillin (Maisonneuve et al., 2013b). In the same study, it was also shown that cells with high expression of RpoS-mCherry had low levels of rrrB P1-gfpunstable. rrrB P1 is repressed by ppGpp (Paul et al., 2004), and it was concluded that both reporters enable identification of persisters by indicating low ppGpp/high expression of toxins. In order to test the dependence of persisters obtained by sorting rrrB P1-gfpunstable cells on toxins, we constructed a strain carrying this reporter in the background of Δ10TA.

We sorted dim cells with a low level of rrrB P1 transcription and measured their survival (Figure 8A). The dim population was ~50 fold enriched in persisters surviving killing by ciprofloxacin compared to the bulk of the population (Figure 8B). In a Δ10TA background, dim cells carrying rrrB P1-gfpunstable are similarly enriched in persisters (Figure 8B). This shows that TAs are not responsible for drug tolerance of cells with low levels of rrrB P1 expression.

We next sought to determine whether the link between rrrB P1 expression and persister formation involves ppGpp. We sorted out rrrB P1-gfpunstable dim cells of a ΔrelAΔspoT deletion strain. The rrrB P1-gfpunstable dim population was enriched in persisters, similarly to the wild-type (Figure 8B). These results indicate that rrrB P1 reports persister levels through a ppGpp-independent mechanism.
Figure 8: *rrnB* P1 promoter activity correlates with persisters and is repressed upon entrance into stationary phase independently of TA interferases and ppGpp.

(A) Exponentially growing cells of MG1655-ASV carrying an *rrnB* P1::gfp unstable transcription fusion were exposed to 1 µg/ml ciprofloxacin for 4h. The antibiotic treated cells were then analyzed by FACS. Dim (5% of the population) and middle (20% of the population) fractions were isolated by cell sorting.

(B) Survival of the dim (Dim) and middle (Middle) was examined by sorting a fixed number of cells onto LB agar for cfu count. Percent of survival for each fraction was determined by comparing cfu count with the total number of sorted cells. Percent of survival of the unsorted control (Unsorted) was determined by comparing cfu count of 4h and 0h of the unsorted culture. Data are averages of at least two independent experiments performed with three biological replicates (n≥6). Error bars represent standard deviation.

(C) Stationary phase *E. coli* MG1655-ASV (WT) and isogenic Δ10TA and ΔrelAΔspoT cultures were diluted 1:100 into LB medium. At each time point, GFP fluorescence was analyzed by FACS to determine the transcription level of *rrnB* P1.
It has been reported that \textit{rrnB P1} is repressed by ppGpp and induced by nucleotide triphosphates, in particular by ATP (Schneider et al., 2002). By adding exogenous adenine or guanine in purine auxotrophs, we were able to raise or lower the cellular ATP concentration and saw a corresponding shift in \textit{rrnB P1} expression in both the wild-type and \(\Delta relA\Delta spoT\) background (Figure 9). This confirmed that \textit{rrnB P1} is a direct ATP sensor independent of ppGpp. These findings led us to reason that persisters may be cells with low ATP levels, which is reported by \textit{rrnB P1}.

![Figure 9: \textit{rrnB P1} senses ATP level independently of ppGpp.](image)

Bactericidal antibiotics kill by corrupting active targets, which require ATP (Davis et al., 1986; Hooper, 2001; Uehara et al., 2009). ATP levels are decreased in stationary phase, and as the culture density increases, the level of persisters rises, reaching 1% once growth ceases (Keren et al., 2004a). Analysis of \textit{rrnB P1}-\textit{gfp}\textsuperscript{unstable} fluorescence over time showed that the fraction of dim cells progressively increases, reaching a maximum at stationary state (Figure 8C). A similar result was observed with \(\Delta 10TA\) and \(\Delta relA\Delta spoT\) strains, again suggesting that the dim cells enriched in persisters have low ATP levels.

**A drop in intracellular ATP causes persister formation**

The level of ATP in stationary phase was lower as compared to exponentially growing cells (Figure 10A), in agreement with published studies (Buckstein et al., 2008). We then decided to emulate
the stationary level of ATP in a growing culture by depleting ATP with arsenate. Cells that had a "stationary" level of ATP tolerated ciprofloxacin and ampicillin (Figure 10B) similarly to stationary cultures. Together, these data suggest that a drop in intracellular ATP concentration is the cause of persister formation. Persisters in a growing population appear to be cells that entered into stationary-like phase early.

**DISCUSSION**

Persisters are formed through redundant mechanisms; screens of knockout libraries in several species have not produced a persisterless strain (De Groote et al., 2009; Hansen et al., 2008; Hu and Coates, 2005; Spoering et al., 2006). TA modules have emerged as a major component responsible for persister formation in *E. coli*. *hipA* was the first gene to be linked to persisters, identified in a screen for

---

Figure 10: Lowering intracellular ATP level leads to increased persister formation

(A) ATP levels were measured in stationary and exponentially growing MG1655 (WT) with firefly luciferase. Cells were treated with arsenate for 30 minutes where indicated.

(B) MG1655 (WT) and isogenic Δ10TA and ΔrelAΔspoT mutants were grown to approximately the same density, then treated with 10 mM arsenate (+AsO4) for 30 minutes where indicated, and challenged with ampicillin or ciprofloxacin for four hours. Results are expressed as % survival by comparison to untreated culture prior to the addition of antibiotic.

Data are averages of at least two independent experiments performed with three biological replicates (n≥6). Error bars represent standard deviation.
hip mutants in the 1980s (Moyed and Bertrand, 1983). A deletion in the hipBA TA locus however produced no phenotype, and this line of inquiry was largely abandoned. With a resurgence of interest in drug tolerance, gain-of-function hipA mutants became a convenient and widely used model to study persisters. We recently reported that hipA mutants conferring 100-1000 fold increase in persisters are present both in commensals and in clinical isolates from patients with UTI, showing that the HipA toxin in these strains becomes not only a biologically relevant, but also the main, component responsible for persister formation (Schumacher et al., 2015). The ability of ectopically expressed interferase (mRNA endonuclease) toxins to produce a similarly large increase in persisters seemed to provide a satisfactory corollary to HipA. Several lines of additional evidence pointed to a role of TA interferases in drug tolerance of E. coli: increased expression in isolated persisters and time-lapse microscopy of cells surviving antibiotic treatment (Keren et al., 2004b; Maisonneuve et al., 2013b; Shah et al., 2006); a sharp decrease in persister levels of a strain deleted in 10 TAs (Maisonneuve et al., 2011); and increased expression during starvation, specifically stringent response. In turn, stringent response was linked to persister formation by several groups and in several species (Korch et al., 2003; Maisonneuve et al., 2013b; Nguyen et al., 2011). The basic experiment was to observe a decrease in the level of persisters in a strain deleted in the two enzymes responsible for the synthesis of the stringent response alarmone ppGpp, relA/spoT. Additional experiments led to a plausible model of persister formation in E. coli: starvation - RelA/SpoT - ppGpp – PPX (inhibition) – polyphosphate - Lon activation - antitoxin degradation - toxin release - inhibition of translation - drug tolerant persister (Maisonneuve et al., 2013b). This model has been largely accepted (Gaca et al., 2015; Helaine et al., 2014; Helaine and Kugelberg, 2014; Holden, 2015; Maisonneuve et al., 2013a).

While we found a TA interferase dependent increase in persister formation under isoleucine starvation conditions, this phenotype was specific to ciprofloxacin. Indeed, the low persister phenotype of the Δ10TA strain seems to be specific to ciprofloxacin under several growth conditions. A recent study in Salmonella showed that a strain lacking six of its TA systems had a low persister phenotype to ampicillin but increased tolerance to ciprofloxacin (Silva-Herzog et al., 2015). This suggests that, similarly to what we find in E. coli, toxins are not involved in Salmonella multidrug tolerance in vitro.
We tested other elements of the standard model by examining the persister phenotype of mutants deleted in the phosphatase or kinase of polyphosphate, *ppx* or *ppk*, and found no phenotype, in agreement with a recent publication (Verstraeten et al., 2015). Similarly, there was no phenotype in cells deleted in the Lon protease, in agreement with our previous report (Theodore et al., 2013), and a strain deleted in *lon* had no effect on persister formation in *vitro* in *Salmonella* Typhimurium closely related to *E. coli* (Helaine et al., 2014; Theodore et al., 2013).

We find that a *relA/spoT* deletion strain makes less persisters, in agreement with previous reports (Maisonneuve et al., 2013b), however, this phenotype is dependent on cell density. Two recent studies have also called into question the link between the stringent response and persister formation. The first found that a *relA* mutant, which cannot synthesize ppGpp in response to amino acid starvation, actually has higher persister levels under some growth conditions (Varik et al., 2016). The second group investigated persister formation in a ppGpp<sup>0</sup> background and found that overexpression of several genes that slow growth increased persister formation. They conclude that many ppGpp independent mechanisms of persister formation exist in *E. coli* (Chowdhury et al., 2016).

Another observation linking stringent response to persisters was based on antibiotic tolerance of individual cells stochastically expressing RpoS, which reports the levels of ppGpp (Maisonneuve et al., 2013b). These cells also had low levels of expression of the ribosomal promoter *rrnB* P1 linked to unstable GFP. *rrnB* P1 is repressed by ppGpp. We reexamined this by sorting cells carrying *rrnB* P1-gfp in the background of a *relA/spoT* deletion. Dim cells were enriched in persisters, showing that *rrnB* P1 is a persister marker which does not depend on of ppGpp.

Our study does not support the notion of TA interferases leading to stochastic persister formation through polyP and *lon* in *E. coli*. At the same time, deletion of any one of a number of TA interferases in *S. Typhimurium* infecting macrophages was reported to produce a dramatic decrease in the level of persisters. Similarly, deleting the *lon* protease had diminished tolerance in that model (Helaine et al., 2014). Why deletion of any single toxin may have a strong effect on tolerance is unclear, but it was suggested that their expression may be linked, so that deleting one affects many. Whether this is indeed the case remains to be established.
Our study has also provided a serendipitous clue to the general cause of persister formation. Having established that the *rrnB* P1 promoter reports persister status independently of ppGpp, we considered its other known effector, ATP. The activity of *rrnB* P1 is positively controlled by ATP (Paul et al., 2004; Schneider et al., 2002), and that is apparently why sorting dim cells in an *rrnB* P1-gfp strain enables isolation of persisters. Interestingly, RpoS is also an ATP reporter (Peterson et al., 2012). Proteolysis of RpoS by ClpPX is inhibited at lower ATP levels. It appears that two persister reporters enable identification of these cells because they respond to ATP as well as ppGpp.

We also find that the fraction of such dim cells increases as the culture progresses from early exponential to stationary state, matching the known phenomenon of persister increase with cell density (Keren et al., 2004a). It appears that persisters in a growing culture are cells that went into a stationary-like state early. Finally, we show that depletion of ATP by arsenate produces tolerance to antibiotics. ATP indeed seems like a good candidate for a general cause of tolerance – bactericidal antibiotics kill by corrupting active, energy-dependent targets. Fluoroquinolones act by converting DNA gyrase and topoisomerase into endonucleases; aminoglycosides cause mistranslation, which produces toxic misfolded peptides; and β-lactams kill cells by forcing a futile cycle of peptidoglycan synthesis (Davis et al., 1986; Hooper, 2001; Uehara et al., 2009). A decrease in target activity will lead to tolerance (Keren et al., 2004a). It is important to note that one specialized mechanism of persister formation, induction of the TisB toxin by the SOS response, leads to a drop in proton motive force, ATP, and drug tolerance (Dorr et al., 2010). Similarly, ectopic overexpression of MazF causes a futile cycle of RNA degradation/synthesis, which leads to a decrease in the energy level and drug tolerance (Mok et al., 2015). Whether stochastic overexpression of a given toxin will be enough to similarly deplete ATP is unclear. Deletion of 10 interferases in our hands causes only a modest drop in tolerance to ampicillin and this was dependent on the medium used, and strong expression of up to eight TA modules by stresses had no effect on persister formation. The exception is isoleucine starvation, which induced the expression of all 10 TA interferases. The increased persister formation after ciprofloxacin, but not ampicillin, challenge was dependent on the TA interferases.

While tolerance by a drop in the energy level explains persister formation, why some cells in a growing culture will have less ATP remains to be established. One possibility is that accidental errors
prevent rare cells from readjusting their metabolism to deteriorating conditions as the density of the culture rises, leading to a drop in ATP and drug tolerance. From this perspective, there is no specialized mechanism of stochastic persister formation. This random error hypothesis (although without the ATP component) was proposed by Neyfakh and co-authors (Vazquez-Laslop et al., 2006).

It appears that there are two different types of mechanisms leading to persister formation in *E. coli*, and probably in other bacteria as well – dedicated persister components, such as TisB or the gain of function mutants of HipA; and a stochastic decrease in ATP, possibly caused by random errors.

**Future Direction**

This study determined that low ATP cells, as reported by *rrnBP1* in a ppGpp<sup>0</sup> background, pre-exist in a population. This low ATP subpopulation is enriched for persisters. Future work will establish a causal link between low ATP and persister formation by determining if lowered ATP decreases antibiotic target activity. One approach will be to measure incorporation of H<sup>3</sup> labeled precursors for RNA, DNA, protein, and peptidoglycan after treatment with an ATP synthase inhibitor. We expect to see decreased incorporation under low ATP conditions showing that the antibiotic targets RNA polymerase, gyrase/topoisomerase, ribosome, and peptidoglycan synthesis are downregulated. Radiolabeled precursor addition is an established method to determine the target of an antibiotic (Ling et al., 2015).

An alternative method is to perform a series of assays to determine the activity of each of the antibiotic targets under low ATP conditions. RNA synthesis will be measured using qRT-PCR; protein synthesis using an inducible GFP system; and the formation of double strand breaks after ciprofloxacin treatment would serve as a proxy for gyrase activity.

A second goal will be to use a direct method to show that low ATP cells are pre-existing in a culture and that these low ATP cells are able to survive and regrow after antibiotic treatment. We will utilize QUEEN, a fluorescent protein that has an ATP binding domain (Yaginuma et al., 2014). The excitation and emission spectra of QUEEN changes when ATP is bound. ATP levels can therefore be determined by calculating the ratio of the two states within a single cell. Yaginuma and colleagues report that they find variation of ATP concentrations between cells within a population, which is what we hope to study. Using fluorescent microscopy, we will monitor the recovery of QUEEN labelled cells after antibiotic
treatment. We expect to find that cells with low ATP before antibiotic treatment survive and are able to regrow after the antibiotic is removed.

Contributions

YS & AB conceived and performed experiments and wrote this chapter. SR provided expertise and feedback and edited this chapter. JD performed experiments. BC provided expertise and feedback and edited this chapter. KL conceived experiments, provided expertise and feedback, and helped to write this chapter.

REFERENCES CITED


